IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Benjamin Eithan Reubinoff, et al.	**Examiner:**	Deborah Crouch
Serial No.:	09/970,543	**Art Unit:**	1632
Filed:	October 4, 2001	**Docket:**	14418Z
For:	EMBRYONIC STEM CELLS AND NEURAL PROGENITOR CELLS DERIVED THEREFROM		

Confirmation No.: 1839

DECLARATION OF Alan Colman UNDER 37 C.F.R. §1.132

Sir:

I, Alan Colman, hereby declare as follows:

1. I am Chief Executive Officer of ES Cell International, 11 Biopolis Way, Helios Block #05-03/04-06, Singapore 138667.

2. I hold a Bachelor of Science (B.S.) Degree in Biochemistry and a Doctorate Degree in Molecular Biology.

3. I have worked a Bachelor of Science (B.S.) Degree in Biochemistry and a Doctorate Degree in Molecular Biology.

4. A true and correct copy of my curriculum vitae is attached hereto as Exhibit A.

5. I have been asked to comment on the differences between human embryonic stem (hES) derived neural progenitor cells (NPCs) disclosed in the above-identified application ("the

'543 application"), and neural stem cells (NSCs) derived from fetal/adult tissues, such as those described in Carpenter (WO 99/11758).

6. I have read the '543 application and the Carpenter application, and am familiar with the contents of both disclosures and the techniques employed therein.

7. The neural progenitor cells (NPCs) disclosed in the '543 application are derived from human embryonic stem (hES) cells. It should be noted that hES cells are not naturally occurring. These cells are considered to be an artifact of an *in vitro* culture system and show properties that are not normally shown by cells of the intact developing embryo or the inner cell mass (ICM) from which they have been derived.

8. Although ES cells exhibit some of the properties of cells of early embryos, no pluripotent cell demonstrates long term self-renewal unless cultured *in vitro*. In fact, pluripotent cells of the early mammalian embryo proliferate only briefly before becoming cells with a more restricted developmental potential. In my opinion, this ability to proliferate indefinitely in an undifferentiated state *in vitro* is consistent with the notion that human ES cells are a tissue culture artifact.

9. The notion that hES cells are the equivalent of early embryonic cells was also called into question in a recent publication co-authored by J. Thomson, the first person to make hES cells. See attached **Exhibit B**, Zwaka and Thomson, *Development* 132: 227-233 (2005). The authors state the following in the summary of the article:

> "Historically, ES cells have been believed to most closely resemble pluripotent primitive ectoderm cells derived directly from the ICM. However differences between ES cells and primitive ectoderm cells have caused developmental biologists to question whether ES cells really have an in vivo equivalent or whether their properties merely reflect their tissue culture environment."

10. The *in vitro* propagation of mammalian neural progenitor cells as neurospheres was first reported in 1992. See, Reynolds and Weiss, *Science* 255: 1707-1710 (1992), attached hereto as **Exhibit C**. These workers were able to extract cells from mouse SVZ or striatum which could be propagated in suspension culture for long periods *in vitro*, as spherical cell clusters, termed neurospheres. The culture medium lacked serum but contained mitogens. The neurospheres were heterogenous in cellular content but when exposed to serum and provided with a substrate, cells within the neurospheres differentiated into neurons and astrocytes. Subsequent work has shown that neurospheres with similar differentiation properties could be obtained from the central nervous system (CNS) in many mammalian species including rats, pigs, and humans. For a long time it was not clear whether neurospheres contained neural stem cells (cells that can indefinitely self renew or, alternatively, differentiate into CNS cell types), in addition to more committed neural progenitor cells. It is now recognized that neurospheres contain a mixture of true neural stem cells and progenitors — although semantically, both cell types are progenitors of their more differentiated progeny including neurons (of different types), astrocytes and oligodendrocytes.

11. It is clear that rodent and human neurospheres are regionally specified. That is, when neurospheres derived from different regions of the brain are differentiated *in vitro*, or after transplantation *in vivo*, it has been found that the cell types that arise in both experimental environments have properties and identities which reflect the region of the brain from which they were originally taken – even after extended passaging. This finding has been documented in Nakagawa et al. (1996) *Development* 122: 1449-2464 (**Exhibit D**); Ostenfeld et al. (2002) *Developmental Brain Research* 134: 43-55 (**Exhibit E**); and reviewed by Temple (2001) *Nature* 414: 112-117 (**Exhibit F**). Thus, neurospheres generated from the striatum, mesencephalon or cortical regions of the embryonic fetal brain gave rise to different cell populations when plated under identical conditions *in vitro*. The interpretation of these observations is that during development, neural stem cells or progenitors acquire subtly different properties according to their time and site of origin in the brain.

12. Hence, in developmental history alone, NPCs developed from hES cells, an artificial source, are quite different to NSCs obtained from an *in vivo* environment, which are

already regionally imprinted and therefore both biased and pre-disposed to differentiate into a neuronal cell type of that region. It is the hES cells, which are a tissue culture artifact, that have been utilized in the '543 application to differentiate in an artificial environment without normal embryological cues to obtain neural progenitor cells. In contrast, the multi-potent NSCs described in Carpenter are isolated from a regionally restricted and defined area, i.e., the forebrain, of a developing fetus. The *in vivo* environment is able to direct differentiation to produce an appropriate cell type in that environment. However, hES cells are cultured in an artificial *in vitro* environment, which is devoid of spatial and physiological cues. The hES cells will have no opportunity to be influenced by a surrounding in vivo environment with appropriate developmental cues as would the cells of the Carpenter application.

13. Interestingly, although the Carpenter application refer to neural stem cells, which were maintained as neurosphere cultures, a later publication which reports some of the work described in the specification states in its conclusion: *"Although these data indicate the presence of an expandable, multipotent population of cells, they do not provide definitive proof of the presence of a human neural stem cell."* Carpenter et al. *Exp Neurology* 158: 265-278 (1999) **(Exhibit G)**, p277, line 3.

14. In my opinion, because of the artificial *in-vitro* nature of human ES cells, it was surprising that hES derived NPCs, when introduced back into an *in vivo* environment, would form stable grafts and integrate into the surrounding neural tissue, as disclosed by the '543 application. Unlike cells derived from much more mature neural tissue, such as the forebrain tissue utilized by Carpenter, that had been subjected to the appropriate neural environmental cues during neural development, the hES derived NPCs have had no spatial relationships with surrounding tissues and environments.

15. In addition to the differences in the developmental history, hES derived NPCs of the '543 application and the NSCs derived from fetal forebrain described in Carpenter, are also distinguished in their expression markers. hES derived NPCs of the '543 application express tyrosine hydroxylase and serotonin after differentiation in simple culture conditions. See, e.g, page 82, line 2 and Figure 28 of the '543 application. In contrast, there is an absence of tyrosine

hydroxylase+ (TH+) neurons (dopaminergic neurons) after differentiation of the "Carpenter" neurospheres, unless cytokines (IL1b) are added (see paragraph 3, page 271, Carpenter et al. (*Exp Neurology* 1999, *supra*). In both cases, identical differentiation conditions are used (addition of serum, removal of mitogens, plating on poly D lysine. The Carpenter cells (following differentiation) appear to be enriched for GABA-ergic neurons. GABA is a non-specific forebrain marker.

16. Furthermore, the Carpenter cells are positive for low levels of GFAP and beta 3 tubulin (as assessed by antibody staining), as indicated in Carpenter's application. See page 3, line 14 of WO 99/11758. In contrast, as shown in the experiment described below, which uses a more sensitive RT-PCR detection method, the NPCs of the '543 application are negative for GFAP. A further difference in the expression profiles of hES derived NPC's and fetal/adult NPC's of the Carpenter invention can be inferred from the stated need for leukemia inducing factor (LIF) in the maintenance/self renewal of the fetal/adult derived NPC's (Carpenter WO 99/11758, Carpenter et al. *Exp Neurology* 158: 265-278 (1999) (**Exhibit G**), Ostenfeld et al. (**Exhibit E**)). In fact, in a later study, Wright et al., 2003 (**Exhibit J**) show that the use of LIF is responsible for the long term growth of the fetal/adult derived NPC's described in Carpenter et al. *Exp Neurology* 158: 265-278 (1999) (**Exhibit G**). They also demonstrate that LIF up-regulates the expression of GFAP in these cells as well as down regulating another 200 genes. In contrast, LIF is not required for long term renewal of the NPCs of the '543 application. It is hard to escape the conclusion that LIF addition during the preparation of fetal/adult derived NPC's greatly exacerbates the intrinsic molecular differences that already exist between NPCs from the two sources.

17. The growth factor is not used in the preparation expansion of the hES derived NPC's of the present invention ("543 application" and Reubinoff et al. *Nat. Biotech* 2001) (**Exhibit H**) and Tamir Ben Hur et al., *Stem Cells* 2005 (**Exhibit I**)). The effect of LIF stimulation is thought to be mediated through its binding to the LIF receptor and activation of the JAK-STAT signaling. One may presume that self renewal of the hES derived NPC's of the present invention do not require external activation of this the pathway, highlighting another

significant difference between these hES derived NPC's and those fetal/adult derived NPC's of the Carpenter invention.

18. In the following experiment, NPCs were prepared from hES cells by ES Cells International, essentially as described in the '543 application. ES Cells International provided these NPCs to Dr. Mahendra Rao at the Stem Cell Section, Laboratory of Neurosciences, Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224, United States for analysis of expression markers. As a comparison, Dr. Rao also prepared progenitor cells from fetal cortex tissue according to the procedure described L.S. Wright et al. 2003 **(Exhibit J)** which is similar to the procedure described in Carpenter's application (WO 99/11758).

19. The results of the analysis were shown in the **Exhibit K**. Clearly, contrary to progenitor cells prepared from adult cortex, the hES-derived NPCs at two passage numbers tested (Passage 0 and Passage 2) do not express GFAP.

20. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that those statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: Alan Bolman Dated: 26 April 2005

EXHIBIT A

Curriculum Vitae

Name	Alan Colman	
Date of Birth	21st October 1948	
Current Address	156 Gibraltar Crescent, Singapore 759588	
Education	Robert Styring Scholar, The Queen's College, Oxford	1967 – 1971
	BA (Hons) Biochemistry, The Queen's College, Oxford	1967 – 1971
Career History	Ph.D. at MRC Laboratory of Molecular Biology Hills Road, Cambridge, UK Supervisor - Dr J B Gurdon FRS	1971 – 1974
	Demonstrator in Developmental Biology Zoology Department, Oxford University, UK	1974 – 1976
	Awarded Brown Junior Research Fellowship, Queens College, Oxford, UK.	1976
	Lecturer in Department of Biological Sciences, University of Warwick, Coventry, UK	1976 – 1983
	Senior Lecturer, Department of Biological Sciences, University of Warwick, Coventry, UK	1983 – 1987
	Professor, Department of Biochemistry, University of Birmingham, UK	1987 – 1993
	Research Director, PPL Therapeutics plc Edinburgh, UK.(part-time)	1988 – 1993
	Research Director, PPL Therapeutics plc Edinburgh, UK (full-time)	1993 – 2002
	Chief Scientific Officer, ES Cell International Singapore	2002 –
	Senior Scientist, Center for Molecular Medicine, Biomedical research Institute, Singapore	2004 –
	Adjunct Professor, Department of Biochemistry, National University of Singapore	2002 –
	Chief Executive Officer, ES Cell International Singapore	2005 –

Membership of Professional Bodies

British Biochemical Society

American Society of Cell Biology

European Molecular Biology Organization (elected 1989)

Membership of Editorial Boards

Editor, Seminars in Cell Biology	1989 – 2003
Trends in Biotechnology	1991 –

Membership of Grant Bodies

UK Wellcome Cell and Molecular Biology Board	1988 – 1991
UK Medical Research Council, Cell & Molecular Medicine Board	1992 – 1996
UK BBSRC Link Cell Engineering Steering Committee	1993 – 2000

Membership of Industrial Bodies

Chairman of the Science Advisory Committee of the UK BioIndustry Association	1994 – 1998

Other Responsibilities

Governor of The Babraham Institute, Cambridge I also sat on their Science Advisory Board and Audit Committee. The Babraham Institute is a highly regarded UK Government – Supported research institute with charitable status	1994 – 2001
Member of the International Advisory Board for the UK Centre for Tissue Engineering	2001 –
National Science and Technology Awards Committee (Singapore)	2003 –
Faculty Advisory Committee, Faculty of Science, National University of Singapore	2003 –

Research Interests & Experience

Academic Period (1971–1992)

My PhD work concentrated on developing the use of the frog (Xenopus) oocyte and embryos for transcription of foreign DNA and in 1975 I published the first evidence that the oocyte machinery could be used in this way (ref 2). Subsequent work by my supervisor and others resulted in the first demonstration of the bone fide transcription, "ex vivo", of an eukaryotic gene.

The use of the frog oocyte and eggs to provide a surrogate test tube was an application which was to dominate my academic career. My primary interest was in using this system to understand generic mechanisms of protein secretion. The frog oocyte is a very large cell which is particularly amenable to a whole range of micromanipulative techniques, including microinjection. Using mRNA microinjection as a means to producing wild type or mutant and mammalian proteins, I was able to test a variety of "secretory" hypotheses prevalent at the time (ref 6).

This system also lent itself to some novel applications, particularly in the area of immunoglobulin assembly and secretion since it was possible to produce the various subunits at different times from each other, at different sites within the oocyte, and with different stoichiometries (ref 21). In two Nature papers in the early eighties, I was able to demonstrate the heterologous expression of a murine antibody in frog oocytes (ref 14), and also, for the first time, I demonstrated that antibodies were functional even as they were folded within the ER—an early demonstration of "intracellular immunisation" (ref 20)

As a result of the above work, I became fascinated with the prospect that the secretory mechanisms I was investigating, figured prominently in the early development of Xenopus, and a lot of my later work was involved in this area.

In order to pursue my objectives regarding protein secretion, I had to develop or refine various technical methods. Prominent amongst there was the use of injected antisense oligonucleotides to remove unwanted endogenous or foreign mRNA's. This resulted in one of the earliest (and most direct) demonstrations of the effectiveness and fidelity of the antisense oligonucleotide methods (ref 48).

Commercial Research Activities and Responsibilities

1 PPL Therapeutics (1987-2002)

Research

During my academic career, I became interested in the commercial exploitation of the biomedical sciences and acted as a consultant to a variety of companies and financial houses, in the period 1981-1987 (e.g. ICI, Amersham, Rothschilds, Prudential, Transatlantic Capital). In 1987, I was invited to be a member of the Science Advisory Board of PPL Therapeutics at its inception that year. The initial mission of this company was to produce therapeutic proteins in the milk of transgenic livestock animals, an endeavour which meshed well with my previous experience with protein secretion. In 1988, as PPL's part-time Research Director, I recruited the first scientists and embryologists to the Company. I became full time Research Director in 1993 (leaving Birmingham University). During the period 1988 - present I was responsible for guiding and implementing PPL's strategic research programme. During this period, PPL grew to around 270 employees with approximately 120 in research.

Research highlights of this period were :

- Birth of Tracy, the highest producer of human protein in the milk of a transgenic mammal. (ref 67). She now resides (stuffed) in the Natural Science Museum in London.
- First report of the complete removal and replacement of a mouse gene with its human homologue. (refs 72,77)
- Establishment of the infrastructure (at PPL Inc, USA) and recruitment of appropriate personnel which has been responsible for the production of the largest number of transgenic founder bovines by any one company.
- Development of a generic method for deriving mammalian embryonic stem cell lines. (ref 83)
- Birth of Dolly (in collaboration with the Roslin Institute) the world's first mammal cloned from an adult cell.
- Birth of Polly (with Roslin Institute), the world's first transgenic, cloned mammal. (ref 85)
- In charge of very successful programme in gene targeting in primary somatic cells which resulted in the births of Diana and Cupid, the world's first livestock with targeted genetic changes (ref 95). I am a named inventor on the patent protecting this work.
- In charge of programme which led to the birth of the world's first cloned pigs. (ref 98)
- Lead the program which recently resulted in production of cloned alpha gal knock out pigs. (Ref 100)
- Developed innovative transgenic and bacterial technology for large scale, cost-effective production of modified, biologically active peptides. (101)
- I was responsible for developing PPL's focus on the use of embryonic stem cells to provide transplantable islets for patients suffering from diabetes. I am a named inventor on a patent covering a method of making ES cells without the need to create and destroy a mammalian embryo.
- Awarded three LINK awards (from three applications) by the UK government and 2 ATP (1999 and 2000) awards from US National Institute of Science and Technology.

As Research Director in charge of over 100 people, I established line management, project management, and a variety of reporting structures within PPL. Before Nov 1999, I had overall responsibility for a research budget of approximately £5-6 million, per annum. In Nov 1999, PPL had to downsize and Research was hard hit. When I left PPL the research team numbered around 50+people.

Product development

Although I had no direct responsibilities for Development, I participated in all the management discussions in getting products to the market and am aware of all the main issues.

Patents

PPL's Patent Department reported to me, and I am knowledgeable about patenting issues and strategies both in the US and Europe.

Collaborations

I was responsible for identifying and negotiating many academic collaborations.

Corporate matters

From 1987 – 1999, PPL was a privately owned company funded mainly by venture capital. In 1996, the company floated on the London Stock Exchange. As an executive director, I was fully involved in all these rounds of funding, receiving a broad education on corporate finance in the process.

2 **ES Cell International (2002-)**

I resigned from PPL on Mar 31 2002 in order to pursue my interests in human embryonic stem cells (hES) with ES Cell International (ESI) where I am now the Chief Executive Officer (CEO). I was Chief Scientific Officer (CSO) from April 2002–Feb 2005. ESI is now concentrating on developing clinically acceptable hES lines as well as devising methods of converting them into either pancreatic beta cells or beating cardiomyocytes in order to address the diabetes and congestive heart failure markets, respectively.

3 **Senior Scientist, Centre for Molecular Medicine 2004–**

Seconded to the Singapore Government's A*STAR Biomedical Research Institute for 20% time to recruit, mentor and lead 10+ scientists in the Regenerative Medicine section.

Presentational skills

I get the opportunity to make countless presentations (many through invitation) at a wide variety of academic, industrial and financial venues. It is an activity I believe I am very good at.

Science and Society

I have often been invited (particularly by EMBO) to act as a spokesperson at meetings on bioethical issues related to cloning, stem cells, and the general use of animals in research . I contributed one article to the new EMBO Reports on the recent controversy regarding the generation and use of human ES cells (99) and another regarding the interface between philosophy and genetic science. (103)

References (Can be supplied on request)

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Publications

1. Colman A, (1974) synthesis of RNA in oocytes of Xenopus laevis during culture in vitro. J. Embryol. Exp.Morph. 32 : 515-532.

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EXHIBIT B

A germ cell origin of embryonic stem cells?

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Summary

Because embryonic stem (ES) cells are generally derived by the culture of inner cell mass (ICM) cells, they are often assumed to be the equivalent of ICM cells. However, various evidence indicates that ICM cells transition to a different cell type during ES-cell derivation. Historically, ES cells have been believed to most closely resemble pluripotent primitive ectoderm cells derived directly from the ICM. However, differences between ES cells and primitive ectoderm cells have caused developmental biologists to question whether ES cells really have an in vivo equivalent, or whether their properties merely reflect their tissue culture environment. Here, we review recent evidence that the closest in vivo equivalent of an ES cell is an early germ cell.

Introduction

Embryonic stem (ES) cells are pluripotent (see Box 1) and can be expanded without limit in vitro (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). It is remarkable that permanent pluripotent stem cell lines can be derived from pre-implantation embryos at all, because, in vivo, pluripotent cells of the early mammalian embryo proliferate only briefly before becoming cells with a more restricted developmental potential. A few years after the initial derivation of mouse ES cells, it was suggested that they be called 'embryo-derived stem cells', a more precise term that would distinguish between these new pluripotent cell lines and cells within the embryo (Rossant and Papaioannou, 1984). However, this term was never adopted, and the extent to which these pluripotent stem cell lines represent any specific embryonic cell type or reflect their artificial tissue culture environment is still an open issue today – two decades later. Elucidating the origin of ES cells is of importance because it may help us to identify genes that are essential for the long-term maintenance of the pluripotent state. It could also assist with the derivation of ES cells from species whose ES cells have proved difficult to isolate. It will also help us to assess how accurately ES cell differentiation reflects events that normally occur in vivo. Here, we review the origin of ES cells, and explore recent evidence that ES cells are closely related to early germ cells.

The historical origins of ES cells: embryonal carcinoma cells

Historically, work with mouse teratocarcinomas paved the way for the derivation of ES cells. These germ cell tumors contain multiple differentiated tissues and undifferentiated stem cells, called embryonal carcinoma (EC) cells (Damjanov and Solter, 1974; Dixon and Moore, 1952; Kleinsmith and Pierce, 1964). Although teratocarcinomas had been known as medical curiosities for centuries (Wheeler, 1983), it was the discovery that male mice of strain 129 had a high incidence of testicular teratocarcinomas (Stevens and Little, 1954) that made these tumors more routinely amenable to experimental analysis. Because their growth is sustained by the persistent EC cell component (Stevens and Little, 1954), teratocarcinomas can be serially transplanted between mice. Eventually, conditions were developed that allowed the culture of EC cells in vitro, establishing them as an in vitro model of mammalian development (Kahan and Ephrussi, 1970).

As pluripotent cells of the intact early embryo proliferate for only a limited period of time, it was not initially obvious whether pluripotent cell lines could be established without undergoing malignant transformation. However, the transplantation of genital ridges or of egg-cylinder-stage embryos into ectopic sites, such as under the kidney capsule of adult mice, gave rise to teratocarcinomas at a high frequency in strains that did not spontaneously produce these tumors (Solter et al., 1970; Stevens, 1970a; Stevens, 1970b). These teratocarcinomas could be serially transplanted between adult mice, depending on whether the EC cell component persisted or differentiated (Solter et al., 1981). If the EC compartment disappears, the resulting tumor develops as a benign teratoma. Indeed, the malignant phenotype of EC cells often depends on the strain of the host mouse, and not on the tumor strain. EC cells injected into mouse blastocysts can contribute to either the normal tissues of the resulting chimera (Brinster, 1974) or, in some cases, to tumors (Rossant and McBurney, 1982). Because the ectopic transplantation of normal peri-implantation embryos can give rise to pluripotent cell lines, the direct derivation of pluripotent cell lines in vitro was attempted without the teratocarcinoma step. The culture conditions that were established to support mouse EC cells, including the use of feeder cell layers, were essentially those used to isolate mouse, and eventually human, ES cells (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998).

One indication that these early EC cell lines may be derived from germ cells (Solter et al., 1970; Stevens, 1967; Stevens, 1970a) came from mouse genital ridge-transplantation experiments. These experiments showed that genital ridges

Box 1. Glossary

Inner cell mass (ICM)
The second lineage of the early embryo that is located inside the blastocyst. It gives rise to all embryonic tissues.

Pluripotency
Refers to the unique ability of cells within the early embryo to differentiate into all cell types.

Primitive ectoderm
The remaining ICM tissue formed during the second differentiation event of embryonic development (also known as epiblast or embryonic ectoderm)

Primitive endoderm
An epithelial layer derived from cells that are in contact with the blastocyst cavity.

Trophectoderm
During the first differentiation event in mammalian development, morula cells segregate into two cell lineages: the first, the trophectoderm, forms the outer layer of the blastocyst. It eventually becomes part of the placenta.

effectively give rise to teratocarcinomas only in a narrow time window (E12.0-12.5). It is around this time that migratory germ cells start arriving in the genital ridge. In the egg cylinder-transplantation experiments, however, the origin of the EC cells was less clear. Primitive ectoderm seemed the most likely candidate for several reasons: because the potential to form teratocarcinomas is lost at the time when primitive ectoderm disappears at E8.5 (Damjanov et al., 1971); because EC cells have phenotypic similarities to primitive ectoderm cells in vivo (Diwan and Stevens, 1976); and because EC cells, when reintroduced into blastocysts, contribute to the same tissues as primitive ectoderm (Brinster, 1974). In addition, when analyzing the earliest stages of teratocarcinoma formation in 129/Sv mouse fetal gonads, Stevens observed clusters of polarized epithelial cells surrounding a central cavity that morphologically resembled primitive ectoderm cells (Stevens, 1983). He also observed that the formation of teratocarcinomas in ovaries included parthenogenic activation of the oocyte, the formation of blastocyst-like structures and the subsequent formation of structures that resembled early egg cylinders, which eventually became disorganized. Isolated transplanted primitive ectoderm itself gives rise to teratocarcinomas (Diwan and Stevens, 1976), but because early germ cells are just appearing at this stage, a germ cell origin cannot be completely ruled out by these experiments.

Are ES cells a tissue culture artifact?

ES cells clearly exhibit some properties that are not normally shown by cells of the intact embryo. For example, although ES cells retain properties of early embryonic cells in vitro, no pluripotent cell demonstrates long-term self-renewal in vivo. Embryonic cells, once brought into tissue culture, are exposed to numerous extrinsic signals to which they never would be exposed to in vivo. ES cells certainly adapt to selective tissue culture conditions and acquire novel functions that allow them to proliferate in an undifferentiated state indefinitely, and, because of this, ES cells are in some sense tissue culture artifacts (Buehr and Smith, 2003; Rossant, 2001; Smith, 2001).

As these changes are inevitable, the issue is not whether ES cells exhibit some properties that merely reflect their tissue culture environment, but rather whether they are most closely related to a specific in vivo cell type in the embryo, or if the influence of the culture environment is so dominant that it is impossible to relate ES cells to a single, in vivo cell type. We will certainly not completely resolve this issue here, but will re-explore the relationship of ES cells to specific early embryonic cell types.

Are ES cells most closely related to primitive ectoderm?

Although ES cell lines are generally derived from the culture of the ICM, some experiments suggest that ES cells more closely resemble cells from the primitive ectoderm. For example, isolated primitive ectoderm from the mouse gives rise to ES cell lines at a higher frequency than does isolated ICM. Moreover, the culture of primitive ectoderm allows the isolation of ES cell lines from mouse strains that have been previously refractory to ES cell isolation (Brook and Gardner, 1997). Indeed, ES cell lines can be derived from single, isolated, mouse primitive ectoderm cells, which is not possible with ICM cells (Gardner and Brook, 1997). Although these experiments suggest that ES cells are more closely related to primitive ectoderm than to ICM, they do not reveal whether ES cells more closely resemble primitive ectoderm or a cell derived from it in vitro.

A maximum of three individual cultured primitive ectoderm cells per embryo have been shown to give rise to ES cell colonies (Gardner and Brook, 1997). This low frequency could have been due to some variability in the potential of primitive ectoderm cells, to some variability in the environment in which they were placed or to damage caused by the dissociation of the primitive ectoderm into individual cells. However, by tracking the expression of the octamer-binding transcription factor 4 (*Oct4*) gene, a marker of pluripotency, in intact cultured ICM/epiblast cells, it was shown that *Oct4* expression was maintained in only a small proportion of outgrowing cells (Buehr et al., 2003), which also suggests that only a minority of primitive ectoderm cells can transit to a new stable, proliferative pluripotent state, and, subsequently, be expanded as ES cells. These results could be due to a requirement for a relatively rare intrinsic or extrinsic stochastic event, or to an inherent heterogeneity of the primitive ectodermal cell population. Recent data indicate that even the earliest ICM is heterogeneous and consists of a mixture of cells that express either *Oct4* or *Gata6* (Rossant et al., 2003), and a similar later heterogeneity could account for the fact that only a minority of primitive ectoderm cells generally give rise to ES cells in culture.

Established mouse ES cell lines express some specific markers of primitive ectoderm at a very low level, if at all (Table 1), such as fibroblast growth factor 5 (*Fgf5*) (Haub and Goldfarb, 1991; Hebert et al., 1991; Rathjen et al., 1999). Culture conditions have been established that convert mouse ES cells into early primitive ectoderm-like cells that express both *Fgf5* and *Oct4* (Rathjen et al., 1999), but these cells fail to form chimeras when injected into mouse blastocysts. Taken together, these results suggest that ES cells are most closely

Table 1. Marker genes expressed in embryonic stem cell (ES), early germ (EGC) and later germ cells (LGC), in the inner cell mass (ICM) and in the primitive ectoderm (PE)*

Gene	Species	ES	EGC	LGC	ICM	PE
Pou5f1 (Pesce and Scholer, 2001)	M	+	+	+	+	+
Nanog (Chambers et al., 2003)	M	+	+	+	+	+
Dppa3 (Saitou et al., 2002)	M	+	+	+	+	+
Ifitm3 (Saitou et al., 2002)	M	+	+	+	+	+
Kit (Horie et al., 1991)	M	+	+	+	–	N/D
DAZL (Clark et al., 2004)	H	+	+	+	–	N/D
Ddx4 (Toyooka et al., 2003)	M	–	–	+	–	–
Akp2 (Chiquoine, 1954)	M	+	+	+	+	+
Zfp42 (Rogers et al., 1991)	M	+	N/D	N/D	+	–
Fgf5 (Haub and Goldfarb, 1991; Hebert et al., 1991)	M	–	N/D	N/D	–	+
Gbx1 (Chapman et al., 1997)	M	+	N/D	N/D	+	–

*Data are based on mouse (M) and human (H) studies, some are preliminary.
+ denotes expression at that developmental stage, – denotes the gene is not expressed
N/D, not done.

related to a subpopulation of primitive ectoderm cells, or to a close derivative of primitive ectoderm cells.

One of the curious species-specific differences between human and mouse ES cells is that human ES cells give rise to trophoblast cells at a high efficiency (Xu et al., 2002), but mouse ES cells do not (Beddington and Robertson, 1989). In the intact mouse embryo, the last cells capable of giving rise to trophoblast cells are early ICM cells, so the failure of mouse ES cells to differentiate into trophoblast is good evidence that they are not the equivalent of early ICM cells (Brook and Gardner, 1997). The differentiation of human ES cells to trophoblast could be explained if they are related to an earlier cell type than mouse ES cells, or if the specification of the trophoblast lineage occurs differently in human embryos. However, a third possibility is that ES cells represent a different cell type altogether. It is therefore worthwhile examining the relationship between ES cells and germ cells.

Germ cells and the primitive ectoderm

In elegant, clonal-fate mapping studies in the mouse (Lawson and Hage, 1994), germ cells were shown to arise from a founder population in the E6.0-6.5 proximal epiblast adjacent to the extra-embryonic ectoderm. These founder cells then pass through the primitive streak and give rise to several extra-embryonic mesodermal lineages and to germ cells. By E7.25, a distinct cluster of ~45 tissue non-specific, alkaline phosphatase (Tnap)-positive germ cells is present at the base of the allantois (Fig. 1) (Ginsburg et al., 1990). The E6.5 distal epiblast, which would not normally contribute to germ cells, will contribute to germ cells if transplanted to a proximal location (Tam and Zhou, 1996), which demonstrates that location and inductive signals, rather than germ plasm determinants, are responsible for the specification of germ cells in mice (Extavour and Akam, 2003). This flexibility suggests that cultured primitive ectoderm cells could spontaneously give rise to early germ cells in culture.

Bone morphogenetic protein 4 (Bmp4) (Lawson et al., 1999) and Bmp8b (Ying et al., 2000) are required for the formation of the proximal posterior extra-embryonic region that gives rise to primordial germ cells (PGCs) and to cells of the allantois in the mouse. The addition of Bmp4 and Bmp8b to distal mouse epiblast cultures increases the formation of cells strongly positive for Tnap (Ying et al., 2001), a marker shared by early germ cells and ES cells. These Tnap-positive cells were interpreted as being germ cells in this study. Recently, BMP signaling has been shown to be important for the self-renewal of mouse ES cells (Ying et al., 2003), and although BMPs are involved in many differentiation decisions in the early embryo, these results do further hint at a relationship between ES cells and early germ cells.

Similarities between germ cells and ES cells

In mice, PGCs migrate and proliferate until ~25,000 are present in the genital ridge at E13.0 (Tam and Snow, 1981). Pluripotent cell lines from pre- and post-migratory (Resnick et al., 1992; Matsui et al., 1992; Shamblott et al., 1998), as well as from migratory (Durcova-Hills et al., 2001), germ cells have been isolated, and these cell lines are termed embryonic germ (EG) cells to distinguish their origin. Mouse EG cell lines are remarkably similar to mouse ES cell lines (Donovan and de Miguel, 2003). During germ cell migration and maturation, however, the somatic status of imprinted genes is progressively erased (Yamazaki et al., 2003), and EG cells isolated at various stages of migration retain some of these differences, such as the reduced methylation of many imprinted genes, including *H19* and *Snrpn* (Hajkova et al., 2002). The analysis of mouse PGCs at E10.5 suggests that methylation erasure has already begun by this time, as supported by studies of the expression of imprinted genes (Yamazaki et al., 2003). This study showed that imprinted genes, such as *H19* and *Snrpn*, exhibit imprinted (somatic) expression patterns in E9.5 PGCs, but by E10.5 have switched to a bi-allelic mode of expression (Yamazaki et al., 2003). Because the genes expressed in ES cells exhibit somatic imprinting patterns (Geijsen et al., 2004), their change in imprinting status suggests that if ES cells are derived from germ cells, this derivation must occur before E9.5.

There is a paucity of known molecular markers that distinguish early germ cells from other pluripotent cells of the early embryo. One marker, Tnap, is strongly expressed by early germ cells and by ES cells, but is weakly expressed by the epiblast and other surrounding embryonic cells (Chiquoine, 1954; Ginsburg et al., 1990). Two new markers for early germ cells, fragilis (Ifitm3 – Mouse Genome Informatics) and Dppa3 (also know as stella or PGC7), have recently been identified that allow the better separation of early germ cell precursors from their differentiated neighboring cells (Saitou et al., 2002).

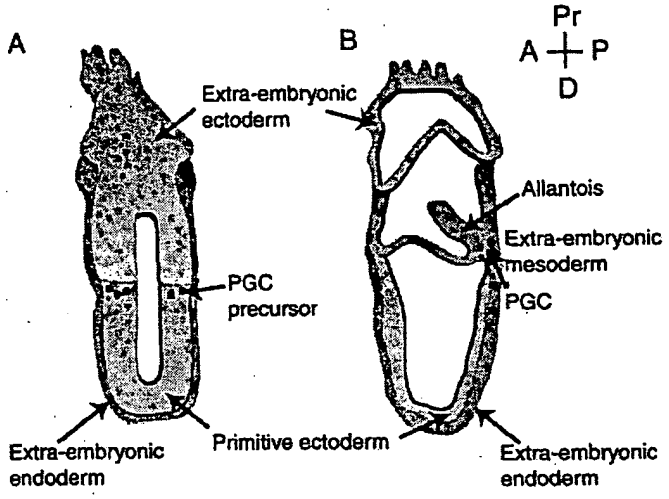

Fig. 1. Early development of the mouse embryo. (A) Six days after fertilization (E6.25), the mouse embryo consists of three layers. The inner cell mass (ICM) cells that are in contact with the blastocyst cavity differentiate into an epithelial layer called the extra-embryonic (primitive) endoderm. The rest of the ICM becomes the epiblast (primitive ectoderm). Primordial germ cells (PGCs, red dots) arise from a cell population in the proximal epiblast adjacent to the extra-embryonic ectoderm. These cells then pass through the primitive streak and give rise to several extra-embryonic mesodermal lineages and to germ cells. (B) By E7.25, a distinct cluster of ~45 tissue non-specific, alkaline phosphatase (Tnap)-positive PGCs is present at the base of the allantois within the extra-embryonic mesoderm (red dots). Once these PGCs are specified, they begin to migrate to the future gonadal anlagen. A, anterior; P, posterior; Pr, proximal; D, distal.

Dppa3 is expressed in pre-implantation embryos and in germ cells (Sato et al., 2002) and has recently been reported to have a role as a maternal transcript in preimplantation embryonic development (Bortvin et al., 2004). Dppa3-positive cells show increased expression of fragilis and remain positive for *Tnap* (*Akp2* – Mouse Genome Informatics) and *Oct4* (Saitou et al., 2002). Once Dppa3-positive PGCs start to migrate, they begin to express additional markers, such as steel factor receptor, followed by markers of more mature germ cells, such as murine vasa homolog (MVH; Ddx4 – Mouse Genome Informatics) (Saitou et al., 2002).

Several recent reports describing the differentiation of mouse ES cells into cells that express markers of mature male and female germ cells (Geijsen et al., 2004; Hubner et al., 2003; Toyooka et al., 2003) are important for our understanding of the origin of ES cells. In each of these reports, germ cell markers were expressed by ES cells themselves, including those, such as Dppa3, that help distinguish germ cells from primitive ectoderm (Table 1). Only the expression of more mature germ cell markers (such as MVH) enabled in vitro-derived germ cells to be distinguished from ES cells themselves. In one study that examined the differentiation of human ES cells into germ cells (Clark et al., 2004), the expression of each of eight genes that are characteristic of early germ cells was detected in human ES cells, but the expression of each of six genes that are characteristic of later germ cells was not detected, strongly suggesting that the expression of the early germ cell-genes was not merely a result of the broadly 'leaky' transcription that is often attributed to ES cells. Using immunocytochemistry, it was also shown that most individual human ES cells in a population express the early germ cell markers stella related (STELLAR) and deleted in azoospermia-like (DAZL), indicating that a minor subset of randomly differentiating cells in a mixed population is not responsible for the expression of germ cell markers in ES cell cultures. Importantly, it was also shown that at least one germ cell-specific gene, *DAZL*, was expressed by human ES cells but not by human ICM. The existing gene expression data, then, are consistent with the idea that the closest in vivo equivalent to ES cells is not the ICM or primitive ectoderm, but an early germ cell.

Some of the properties of ES cells, however, suggest that they are not merely the equivalent of early germ cells. For example, the earliest PGCs do not self-renew for prolonged periods of time, but instead begin a series of maturation steps, beginning with germ cell migration and ending in the highly specialized development of sperm or egg (Wylie, 1999). Although ES cells can differentiate into more mature germ cells in vitro, they do so relatively inefficiently. Indeed, the ability to colonize the germline of chimeras is one of the most easily lost properties of ES cells. If ES cells most closely represent early germ cells, it is unclear why they are not better at giving rise to more mature germ cells. In addition, isolated PGCs have never been demonstrated to contribute to chimeras when injected into blastocysts, so an exact equivalence to ES cells is unlikely.

Because a comprehensive and comparative analysis of the transcriptomes of isolated ICM, primitive ectoderm and early germ cells has not yet been reported, it is not yet clear how much the particular repertoire of genes expressed by ES cells represents an early germ cell, another specific in vivo cell type, a response to the tissue culture environment, or a combination of all three. If the ICM and primitive ectoderm are inherently heterogeneous, transcriptome analysis may need to be carried out at the single-cell level to ultimately understand these relationships. However, at the moment, the greatest concordance of known markers appears to be between ES cells and early germ cells.

Conclusions

We hypothesize that ES, EC and EG cells represent a family of related pluripotent cell lines, whose common properties reflect a common origin from germ cells (Fig. 2). Although a more detailed transcriptional analysis could ultimately refute the proposed relationship between ES cells and early germ cells, we hope this idea will at least help to stimulate a healthy

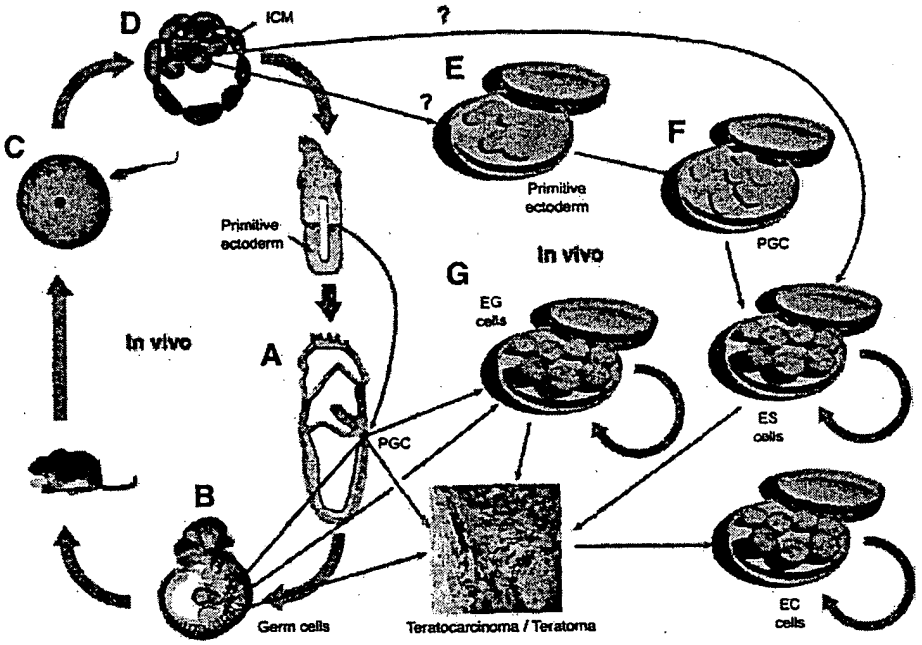

Fig. 2. Germ cell propagation in vitro and in vivo. (A-C) The germ cell cycle in the mouse. (A) Primordial germ cells (PGCs) appear at E7.25 as a small group of cells (red) in the extra-embryonic mesoderm. (B) After E8.5, PGCs start to migrate to the gonadal anlagen and contribute (C) during puberty to oocytes or sperm. (D) Embryonic stem (ES) cells are in vitro derivatives of inner cell mass (ICM) cells. (E,F) The formation of ES cells occurs either directly from the ICM/primitive ectoderm (E) or according to our hypothesis, through in vitro differentiation of ICM outgrowth into primitive ectoderm, then into extra-embryonic mesoderm and finally into PGCs (F). (G) PGCs that form in vivo (A-C) give rise to embryonic germ (EG) cells in vitro. Germ cells, PGCs, ES and EG cells are all capable of forming teratomas and teratocarcinomas. ES and EG cells can reintegrate into the normal embryo after injection into the blastocyst (not shown). Circular green arrows denote unlimited self-renewal.

re-evaluation of what is actually being studied when ES cells differentiate in vitro.

What is the relevance of a putative close relationship between ES cells and early germ cells? One prediction of this hypothesis is that at least some of the germ cell-specific genes expressed by ES cells, and not by primitive ectoderm cells, are essential for the long-term maintenance of the pluripotent state. If true, then it should be possible to generate knockout mice to identify genes that are essential for the specification or maintenance of PGCs, which are also essential for the derivation of ES cell. A related prediction of the hypothesis is that genes that are responsible for increasing susceptibility to spontaneous germ cell tumors should increase the efficiency of ES cell derivation. It is interesting, for example, that in species where teratocarcinomas occur at a clinically significant frequency, such as in mouse and human, ES cells have been successfully derived, whereas in species where teratocarcinomas are exceedingly rare, such as the rat, ES cells have proven difficult to derive. Understanding basic species differences in the specification or maintenance of early germ cells could allow the derivation of ES cells from species that have been hitherto resistant to the isolation of ES cells, such as the rat (Buehr et al., 2003).

Another implication of our hypothesis is that when looking for evolutionary clues to understand the pluripotent state, the comparative germ cell literature will be the most instructive. In a species such as the zebrafish, which has a germ plasm that strictly separates germ cells from somatic cells, it makes sense that pluripotent cell lines that can contribute to the germline in chimeras (Ma et al., 2001) would have to be derived from germ line-lineage cells.

Another prediction arising from the hypothesis that ES cells most closely represent early germ cells is that the very earliest events of ES cell differentiation into somatic and extra-embryonic lineages will not accurately reflect events that normally occur in vivo. The idea that ES cells represent an in vitro equivalent to the ICM, however, is firmly entrenched and continues to strongly influence our thinking about these cells. When examining the differentiation of ES cells in vitro, the pervasive mental image is of a forward progression that recapitulates normal embryonic events. For example, one thinks of ICM cells progressing to primitive ectoderm cells, then to neural ectoderm cells, and finally to more specialized neural cell types. If ES cells most closely represent early germ cells, this mental image needs revision, as the earliest transition would appear to be more 'lateral' or even 'backward' than 'forward'. It will be illuminating to define each of the distinct transitions that ES cells can make in a single step and to determine how much these initial transitions resemble in vivo or artificial differentiation. If ES cells really represent early germ cells, the initial events in differentiation would be expected to be transitions that do not normally occur in intact

embryos, except, perhaps, when the transition is to more mature germ cells.

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EXHIBIT C

Generation of Neurons and Astrocytes from Isolated Cells of the Adult Mammalian Central Nervous System

Brent A. Reynolds; Samuel Weiss

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change accompanying phosphorylation of Ser^{103} occurs at a distant, voltage-sensing, transmembrane region. Our finding that the greatest effect of PDD occurs at small depolarizations indicates that phosphorylation has a physiological function because K^+ conductance would be reduced at potentials close to rest.

The change in voltage sensitivity associated with phosphorylation strongly suggests that the protein is a channel subunit. This conclusion agrees with the finding that mutations in the hydrophobic domain can alter ion selectivity (*9*). Furthermore, the results of our site-directed mutagenesis support a structural model for the I_{sK} protein in which the NH_2-terminus is outside the cell, there is a single transmembrane domain, and the COOH-terminus is within the cell (Fig. 1). Thus, the alteration in the voltage dependence of I_{sK} observed in the mutation at Ser^{69} is consistent with that residue lying within the membrane, and the effects of the mutation at Ser^{103} show that this amino acid must be intracellular.

It remains uncertain which K^+ currents recorded from mammalian cells result from activation of this molecular species of the K^+ channel. Sensitivity to phorbol esters may be helpful in the identification of this channel as it occurs in mammalian cells.

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11. To obtain a clone encoding I_{sK}, we used oligonucleotides specific for sequences flanking the I_{sK} coding region (*5*) and containing synthetic restriction endonuclease sites in the polymerase chain reaction with reverse-transcribed rat kidney RNA as substrate. The reaction product was purified through an agarose gel, eluted, and directionally subcloned into the vector pS$^-$ (J. P. Adelman, unpublished results). After transformation into *Escherichia coli* JM101, single-stranded DNA was retrieved by superinfection, and this material was used to verify the I_{sK} coding sequence. RNA transcripts encoding I_{sK} were synthesized in vitro from this plasmid and injected into *Xenopus* oocytes. The methods for injection, maintenance, and recording from oocytes have been described [M. J. Christie, J. P. Adelman, J. Douglass, R. A. North, *Science* 244, 221 (1989)]. The superfusing solution used during recordings contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes (pH 7.4; at 20° to 22°C). Site-directed mutagenesis was as carried out as described [R. S. Hurst *et al.*, *Mol. Pharmacol.* 40, 572 (1991)].
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Generation of Neurons and Astrocytes from Isolated Cells of the Adult Mammalian Central Nervous System

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Neurogenesis in the mammalian central nervous system is believed to end in the period just after birth; in the mouse striatum no new neurons are produced after the first few days after birth. In this study, cells isolated from the striatum of the adult mouse brain were induced to proliferate in vitro by epidermal growth factor. The proliferating cells initially expressed nestin, an intermediate filament found in neuroepithelial stem cells, and subsequently developed the morphology and antigenic properties of neurons and astrocytes. Newly generated cells with neuronal morphology were immunoreactive for γ-aminobutyric acid and substance P, two neurotransmitters of the adult striatum in vivo. Thus, cells of the adult mouse striatum have the capacity to divide and differentiate into neurons and astrocytes.

THE GENERATION OF NEURONS IN the mammalian central nervous system (CNS), with few exceptions, occurs during early development (*1*). Mitogenic growth factors, such as basic fibroblast growth factor (bFGF) and nerve growth factor (NGF), may participate in the production of CNS neurons (*2, 3*). Epidermal growth factor (EGF) is a powerful mitogen of numerous non-neuronal cells and enhances wound healing and tissue regeneration in various adult organs such as skin, liver, and intestinal epithelium (*4*). In the CNS, mitogenic and trophic actions of EGF on embryonic and early postnatal cells indicate its importance in neuronal development (*5*). The demonstration of EGF- and EGF receptor–immunoreactivity in the adult rodent and human CNS (*6*) prompted us to examine whether EGF-responsive cells could be isolated from the adult mouse CNS.

The striata of 3- to 18-month-old adult mice were enzymatically dissociated and plated in serum-free culture medium containing 20 ng of EGF per milliliter. Cells were seeded in 35-mm-diameter culture dishes (1000 viable cells per plate) in the absence of supplementary substrate or adhesion factors (*7*). After 2 days in vitro (DIV) most of the cells had died; however, 15 ± 2 cells per plate (n = 4 independent culture preparations; the striata of two adult mice were pooled in each of the four experiments) were undergoing cell division (Fig. 1A). Cell division continued for an additional 2 to 3 DIV (Fig. 1, B and C), after which the proliferating clusters of cells detached and formed (6 to 8 DIV) a sphere of proliferating cells (Fig. 1D). Cell division and proliferation were not observed in the absence of EGF, nor were they mimicked by bFGF (20 ng/ml), platelet-derived growth factor (20 ng/ml), or NGF (100 ng/ml). In addition, if cells were seeded on a substrate that had been treated with poly-L-ornithine, proliferation was not observed in the presence of EGF. These findings suggest that the presence of both EGF and a nonadhesive substrate is required to initiate cell division of these isolated adult striatal cells.

To assess the antigenic properties of cells within these 6- to 8-DIV spheres, we transferred them to poly-L-ornithine–coated cover slips, allowed them to adhere for 1 hour, and processed them for indirect immunocytochemistry (*8*). Virtually all cells in the spheres were immunoreactive for nestin (Fig. 1, E and F; n = 8 independent culture

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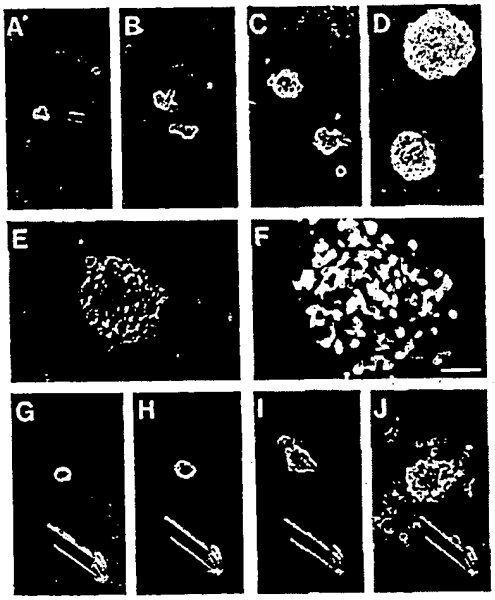

Fig. 1. EGF-induced proliferation of cells isolated from the adult mouse striatum. (**A**) After 2 DIV, cells that had undergone cell division were first observed. Cell division continued at 3 (**B**) and 4 (**C**) DIV, although dividing cells (beginning to form a cluster) migrated slowly across the substrate. (**D**) After 6 to 8 DIV, spheres of cells lifted off the substrate and floated in suspension. Line in substrate (A through C) serves to identify the field. (**E**) One hour after plating onto poly-L-ornithine, a 6-DIV sphere attached to the substrate. (**F**) The cells in (E) were immunostained with antibody to nestin; virtually all cells were immunoreactive for nestin. (**G** through **J**) Single cells, derived from dissociated 6- to 8-DIV spheres, were plated in single wells of a 96-well plate. A large, hypertrophic cell after 2 DIV (G) began to divide and form a cluster of cells during the subsequent 3 (H), 4 (I), and 6 (J) DIV. Scratches in substrate serve to identify the field. Scale bars: (A through D) bar in (D) denotes 50 μm; (E), 50 μm; (F), 25 μm; (G through J) bar in (J), 50 μm.

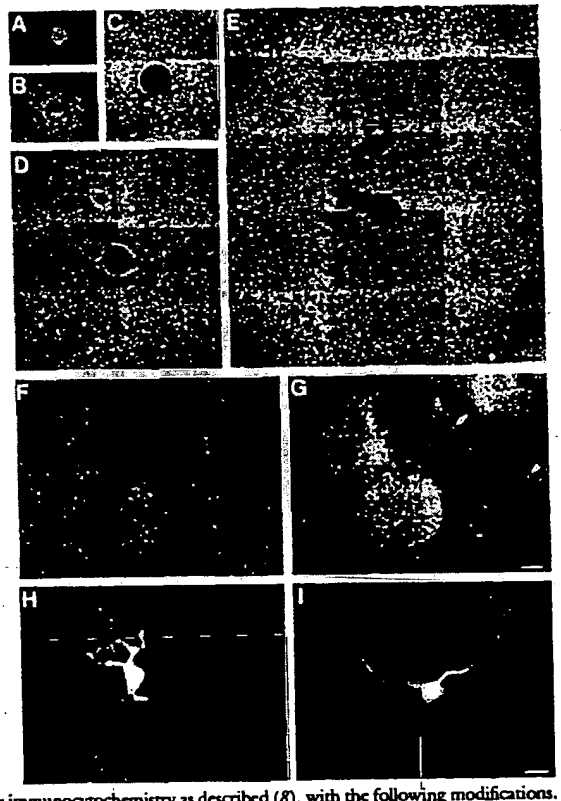

Fig. 2. NSE- and GFAP-positive cells generated from a single sphere of EGF-induced, undifferentiated adult striatal cells. (**A**) A single sphere immediately after plating. (**B** through **D**) The same sphere after 1 (B), 3 (C), and 14 (D) DIV, respectively. (**E**) After 21 DIV, the transferred sphere and associated cells were fixed and processed for dual-antigen immunocytochemistry. (**F** and **G**) Fluorescence micrographs of the portion of field delineated in (E), after dual-labeling of the same field with antibody to GFAP (F) and antiserum to NSE (G), respectively. Arrowhead and arrow indicate NSE-positive cells enlarged in (H) and (I), respectively. Scale bars: (A through E) bar in (E) denotes 250 μm; (F and G) bar in (G), 100 μm; (H and I) bar in (I), 10 μm. Single spheres (6 DIV), generated as described (*7*), were transferred with a pipette to poly-L-ornithine–coated glass cover slips in the base of 12-well culture dishes and cultured in serum-free medium containing EGF (which was replenished twice weekly thereafter). After 21 DIV, cover slips were processed for immunocytochemistry as described (*8*), with the following modifications. The primary antibodies (applied together) were a mouse monoclonal antibody to GFAP (1:100; Boehringer Mannheim) and rabbit antiserum to NSE (1:300; Dakopatts, Carpenteria, California), and the secondary antibodies (applied together) were fluorescein-conjugated goat antibody to mouse IgG (1:100; Jackson Immunochemicals) and rhodamine-conjugated goat antibody to rabbit IgG (1:100; Jackson Immunochemicals).

preparations), an intermediate filament found in neuroepithelial stem cells (*3, 9*). In contrast, cells in the 6- to 8-DIV spheres were not immunoreactive for neurofilament (168 kD), neuron-specific enolase (NSE), and glial fibrillary acidic protein (GFAP) (*10*). To determine whether cells within the 6- to 8-DIV spheres could continue to proliferate in secondary cultures, spheres were mechanically dissociated and replated as single cells in the wells of 96-well plates (*11*). In the presence of EGF, single cells proliferated and formed new spheres (Fig. 1, G through J); the majority of cells within these secondary spheres were also immunoreactive for nestin (*10*). When 200 to 250 of these cells were plated in a 35-mm dish, in the presence of EGF and in the absence of supplementary substrate or adhesion factors, 67 ± 4% (n = 3 independent culture preparations) of the cells formed new spheres. As above, if EGF was omitted from the serum-free culture medium, proliferation was not observed. In addition, when EGF was removed from the medium after proliferation had been initiated, no further proliferation was observed. These findings suggest that in vitro conditions may be established for the continual proliferation of undifferentiated cells originally derived from the adult mammalian CNS.

We next examined whether, given a suitable substrate, cells generated from EGF-induced spheres would develop the morphological and antigenic properties of the principal cell types of the CNS. Single 6- to 8-DIV spheres were transferred with micropipettes to poly-L-ornithine–coated glass cover slips (in 12-well plates) and cultured in the continued presence of EGF-containing, serum-free medium (Fig. 2A). During the subsequent 21 DIV, cells were observed migrating from the sphere, which continued to proliferate in the presence of EGF (Fig. 2, B through E). After 21 DIV, the proliferating sphere and cells that had migrated from the core were processed for dual-antigen, indirect immunocytochemistry; both GFAP- (Fig. 2F) and NSE- (Fig. 2G) immunoreactive cells were present. These findings were reproduced in eight independent culture preparations. Incorporation of bromodeoxyuridine (1 μM, applied between 10 and 13 DIV) into NSE-immunoreactive cells (Fig. 3, A and B) supports our conclusion that these cells were born during the culture period. The EGF-induced pattern of proliferation precluded an accurate count of the total number of cells produced. Nevertheless, in the GFAP plus NSE dual-labeling experiments, 61 ± 7% of the labeled cells were immunoreactive for GFAP, whereas 39 ± 5% were immunoreactive for NSE (as assessed by counting 827 immunoreactive

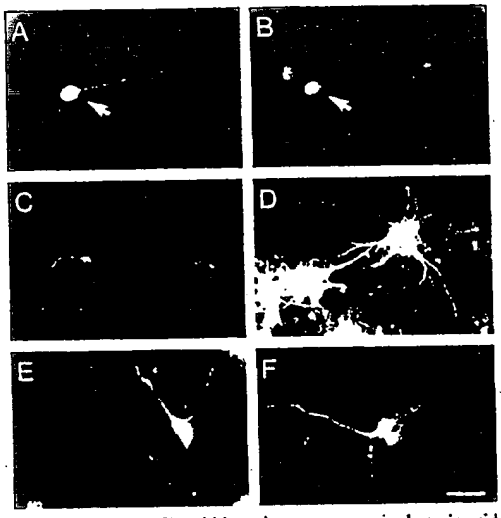

Fig. 3. Morphology and phenotype of cells generated by EGF-induced proliferation of undifferentiated adult striatal cells. Fluorescence photomicrographs of cells in cultures of single, EGF-generated spheres after 21 DIV on poly-L-ornithine. (**A**) An NSE-immunoreactive cell (arrowhead) that had incorporated bromodeoxyuridine (**B**), the distinct morphology of neurofilament (168 kD)-immunoreactive (**C**) and GFAP-immunoreactive (**D**) cells, and cells with neuronal morphology that were immunoreactive for γ-aminobutyric acid (**E**) and substance P (**F**). Bar, 20 μm. Culture and immunocytochemistry of single spheres were carried out as described (*7, 8*) with the following modifications. Primary antibodies used included mouse monoclonal antibody to bromodeoxyuridine (undiluted; Amersham), monoclonal antibody to neurofilament (168 kD) antibody (1:50; clone RMO 270), rabbit antiserum to γ-aminobutyric acid (1:3000; Incstar, Stillwater, Minnesota), or rabbit antiserum to substance P (1:100; Incstar).

cells in four independent culture preparations). The majority of cells not immunoreactive for GFAP or NSE were immunoreactive for nestin (*10*).

In EGF-generated cultures, the NSE-immunoreactive cells generally had small, rounded somas with fine processes (Figs. 2, H and I, and 3A). In contrast, GFAP-immunoreactive cells were stellate, with large somas and several thick processes (Fig. 3D). Cells with rounded soma and long, thin processes also were immunoreactive for neurofilament (168 kD) (Fig. 3C). To determine whether these cells contained CNS neurotransmitters, we tested for the presence of amino acids, biogenic amines, and neuropeptides with indirect immunocytochemistry. Cells with neuronal morphology that were immunoreactive for γ-aminobutyric acid (Fig. 3E) and substance P (Fig. 3F) were present throughout 21-DIV cultures of spheres and associated cells (n = 3 independent culture preparations); these are two of the major neurotransmitters of the adult striatum in vivo (*12*). In contrast, these cultures did not contain cells that were immunoreactive for glutamate, serotonin, tyrosine hydroxylase, methionine-enkephalin, neuropeptide Y, or somatostatin (*13*), the latter three being present in the adult striatum. The reason for the restricted expression of phenotypes is unknown, but it is possible (i) that EGF-responsive progenitor cells are limited to produce only γ-aminobutyric acid– and substance P–containing cells or (ii) that other phenotypes may appear at different times or under different culture conditions.

Our results demonstrate that EGF induces the proliferation of a small number of cells, isolated from the striatum of the adult mouse brain, that produce clusters of cells with antigenic properties of neuroepithelial stem cells. Under appropriate conditions these cells can be induced to differentiate into astrocytes and neurons with phenotypes characteristic of the adult striatum in vivo. We have recently isolated an EGF-responsive, multipotent stem cell from the embryonic striatum that exhibits a pattern of proliferation and differentiation that is indistinguishable from that described in this study (*10*). Taken together, these findings suggest that a population of embryonic stem cells may survive in the adult brain in a dormant, nonproliferative state. Alternatively, these cells may be similar to a population of subependymal cells in the dorsolateral region of the lateral ventricle that proliferates in the adult mouse, although the fate of half of the progeny is death (*14*). Neurogenesis in the mouse striatum is complete after the first few postnatal days (*15*). The inability of these stem cells to continue to proliferate in vivo may be due to inhibitory influences of the adult mammalian CNS. Inhibition of initial EGF-induced proliferation on plates treated with poly-L-ornithine suggests that the substrate may be one of these inhibitory influences.

Although neuroepithelial stem cells may be manipulated in vitro after isolation from fetal brain tissue (*16*), our findings indicate that the adult brain may serve as an alternative source. The ability to induce EGF-responsive stem cells to proliferate in suspension in vitro, and to reinitiate proliferation in a large percentage of the progeny, can provide a plentiful source of undifferentiated CNS cells from the adult for studies of their basic properties and for use in experimental models of autologous or heterologous CNS transplantation. Identification of factors that induce or inhibit the in situ proliferation and differentiation of these cells may allow for their eventual manipulation in the intact adult mammalian CNS to replace cells lost to injury or disease.

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8. After 6 DIV, floating spheres were transferred with wide-bore pipettes onto poly-L-ornithine (15 μg/ml) (Sigma) –coated glass cover slips in 24-well culture dishes. After 1 hour, the medium was replaced with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, followed by three 10-min rinses in PBS. Attached spheres were incubated with a rabbit polyclonal antiserum directed against nestin (Rat 401). Cover slips were incubated in antibody to nestin (1:1500 in PBS/0.3% triton-X-100/10% normal goat serum) for 2 hours at 37°C. Cover slips were rinsed three times (10 min) with PBS and incubated with rhodamine-conjugated goat antibody to rabbit immunoglobulin G (IgG) (1:100; Jackson Immunochemicals) for 30 min at room temperature. After three final washes in PBS, coverslips were mounted with Fluorsave (Calbio-

chem) on glass slides and viewed and photographed with a Nikon Optiphot photomicroscope. Omission of primary antiserum or its replacement with non- or preimmune serum resulted in no detectable staining.
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Evidence for a Computational Distinction Between Proximal and Distal Neuronal Inhibition

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Most neurons have inhibitory synapses both "proximally" near the spike-initiating zone and "distally" on dendrites. Although distal inhibition is thought to be an adaptation for selective inhibition of particular dendritic branches, another important distinction exists between proximal and distal inhibition. Proximal inhibition can attenuate excitatory input absolutely so that no amount of excitation causes firing. Distal inhibition, however, inhibits relatively; any amount of it can be overcome by sufficient excitation. These properties are used as predicted in the circuit-mediating crayfish escape behavior. Many neuronal computations require relative inhibition. This could partly account for the ubiquity of distal inhibition.

MOST CENTRAL NEURONS RECEIVE inhibitory inputs on their basal dendrites or somata, where they are well situated to shunt distally generated excitatory postsynaptic potentials (EPSPs) before they spread to the axonal spike initiating zone. Such "proximal inhibition" was discovered first, but it is now known that distal dendrites also commonly receive extensive inhibitory as well as excitatory inputs (*1*). A reason often proposed for such "distal inhibition" is that it would allow for selective inhibition of particular dendritic branches (*2*). While studying inhibitory control of the lateral giant (LG) command neurons for crayfish tail-flip escape behavior, we have come to appreciate a different and perhaps more generally important reason for distal inhibition.

Two kinds of suppression of LG escapes are known: (i) Escape cannot be initiated while an escape response is in progress. The responsible suppressive influence is called recurrent inhibition (*3*). Because initiating a new response while another is in progress would be maladaptive, recurrent inhibition should be absolute: No matter how strong the provocation, the inhibition should prevent a response. (ii) The likelihood of escape is reduced when crayfish are feeding or are restrained so that escape would be ineffectual (*4*). The responsible process, tonic inhibition, is strategic in nature: Although ignoring a modest threat to continue feeding may be adaptive, sufficient provocation should still cause escape. Thus, it should be possible to override tonic inhibition.

Both recurrent and tonic inhibition are known to be directed to the LG neuron itself and thus might be produced by the same inhibitory neurons. However, a consideration of the way excitation and inhibition interact suggests that proximal inhibition best confers the absolute suppression required for recurrent inhibition, whereas distal inhibition can more readily be overcome by strong excitation, as required for tonic inhibition.

The reason is illustrated in Fig. 1, which shows predictions from a steady-state, two-compartment model of LG (equivalent circuit insets). Attenuation of EPSPs from distal to proximal compartment was by a factor of 10, consistent with known physiology and anatomy. The top curve in Fig. 1, A1 and A2, shows the depolarization produced by excitation in the absence of inhibition. Depolarization increases with increasing excitation until the EPSP at its distal origin approaches the excitatory synaptic reversal potential and saturates. Thus, the curve plateaus.

Proximal inhibition decreases EPSP amplitude by a nearly constant factor independent of the amount of excitation (Fig. 1, A1 and B1). Thus, if the inhibitory strength were sufficient to reduce the plateau of the curve in Fig. 1, A1, below the critical firing level of the proximal compartment (for example, the dashed line), then no amount of additional excitation would fire the cell. In other words, the inhibition would be absolute, as required for recurrent inhibition.

Distal inhibition behaves differently. Because it reduces EPSPs in the distal compartment itself, it tends to counter the excitatory saturation, allowing excitatory levels that in the uninhibited situation would produce no further potential change to do so, until the inhibition is overcome and excitation again saturates. Thus, any amount of distal inhibition can be overcome by further excitation, as reflected in Fig. 1, A2, by the fact that all curves crossed the critical firing level with strong enough excitation. Consequently, distal inhibition can be overridden, as required for tonic inhibition. We refer to the different functional properties of inhibition conferred by proximal and distal synapses as "absoluteness" and "relativity," respectively.

These arguments imply that recurrent inhibition of LG should operate proximally, whereas tonic inhibition should be distal. Is this the case? To evaluate this, we compared the conductance increases produced by recurrent and tonic inhibition in the proximal dendrites of LG (Fig. 2A). If the hypothesis is correct, the proximal conductance increase associated with recurrent inhibition should be much greater than that associated with comparable amounts of tonic inhibition.

Recurrent inhibition was produced by firing the medial giant neurons, which cause an escape response without LG neuron involvement (*5*); inhibition was measured near its maximum, about 10 ms after medial giant firing (*3*) (Fig. 2, B1). We measured recurrent inhibition in the absence of tonic inhibition by isolating the abdominal ner-

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EXHIBIT D

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Roles of cell-autonomous mechanisms for differential expression of region-specific transcription factors in neuroepithelial cells

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SUMMARY

Although a number of genes have been found to have restricted expression domains in the embryonic forebrain and midbrain, it remains largely unknown how the expression of these genes is regulated at the cellular level. In this study, we explored the mechanisms for the differential expression of region-specific transcription factors in neuroepithelial cells by using both primary and immortalized neuroepithelial cells from the rat brain at embryonic day 11.5. We found that differential expression patterns of *Pax-3*, *Pax-5*, *Pax-6*, *Dlx-1*, *Dlx-2*, *Emx2*, *Otx1* and *Dbx* observed in vivo were maintained even when the cells were isolated and cultured in vitro, free from environmental influences. Furthermore, in response to Sonic hedgehog, which is a major inductive signal from the environment for regional specification, neuroepithelial cells that maintain distinct regional identities expressed different sets of ventral-specific genes including *Islet-1*, *Nkx-2.1* and *Nkx-2.2*. These results suggest that certain cell-autonomous mechanisms play important roles in regulating both environmental signal-dependent and -independent expression of region-specific genes. Thus, we propose that use of the in vitro culture systems we describe in this study facilitates the understanding of regulatory mechanisms of region-specific genes in neuroepithelial cells.

Key words: neural development, regional specification, neural stem cells, neuromere, homeobox gene, rat

INTRODUCTION

The mammalian central nervous system is the center of higher cognitive functions. Its generation involves a number of events including neural induction, patterning of the neural plate, commitment and differentiation of neurons and glia, and establishment of neuronal and glial connections. These events collectively contribute to the generation of neural cell diversity. However, the molecular mechanisms controlling this complex process have just begun to be elucidated.

An important aspect of early development of the brain is the regional specification of neuroepithelial cells (Jessell and Dodd, 1992; Ruiz i Altaba, 1994; Lumsden and Graham, 1995). For example, it is well established that the vertebrate hindbrain neuroepithelium is composed of discrete anatomical as well as functional units called rhombomeres, which serve as a framework for subsequent regional specification. In chick embryos, the cranial motor nerve nuclei are derived from adjoining pairs of rhombomeres (Lumsden, 1990), which indicates that neuroepithelial cells in each rhombomere are fated to give rise to particular types of neurons. Searches for possible molecular correlates of the rhombomeres have succeeded in identifying many putative regulatory genes that show segment-specific expression domains. These include the *Antennapedia* class of homeobox-containing (*Hox*) genes (Wilkinson et al., 1989a) and the zinc-finger gene *Krox-20* (Wilkinson et al., 1989b). The boundaries of these expression domains have been shown to restrict the mixing of neuroepithelial cells (Birgbauer and Fraser, 1994) as well as the intercellular movement of small molecules via gap junctions (Martinez et al., 1992).

Although there have long been controversies over the early organization of more anterior parts of the brain, i.e. the forebrain and midbrain, the recent discoveries of genes that are expressed in a region-specific manner along with refined morphological studies have revealed that the vertebrate forebrain, like the hindbrain, can also be divided into discrete domains (Bulfone et al., 1993; Figdor and Stern, 1993; Puelles and Rubenstein, 1993; Macdonald et al., 1994). Bulfone et al. (1993) compared the expression domains of the *Dlx-1*, *Dlx-2*, *Gbx-2* and *Wnt-3* genes in the embryonic day (E) 12.5 mouse forebrain and concluded that the developing forebrain can be

divided into six transverse domains called prosomeres, and that each prosomere can be subdivided further into longitudinal domains. These proposed domain structures matched the neuromeric model of the forebrain organization based on morphological studies (Puelles et al., 1987). Analyses of expression domains of other genes from different families including the paired-domain-containing *Pax* family (Stoykova and Gruss, 1994) and the homeobox-containing *Otx*, *Emx* and *Nkx* families (Simeone et al., 1992a,b; Guazzi et al., 1990; Price et al., 1992; Shimamura et al., 1995) have provided further evidence for their hypothesis (see Boncinelli et al., 1993 and Rubenstein et al., 1994 for reviews). Studies of chick and zebrafish embryos revealed that such gene expression boundaries partially restrict cell mixing and are correlated with the positions of early generated neurons and their axonal tracts (Figdor and Stern, 1993; Wilson et al., 1993; Macdonald et al., 1994; Guthrie, 1995). Although the functions of these region-specific genes remain to be elucidated, some of them are expressed in specific populations of postmitotic neurons at later stages of development. Since many of these region-specific genes encode transcription factors, they are likely to serve as important regulators in the genetic network leading to the generation of particular neural cell types. Therefore, it is reasonable to expect that neuroepithelial cells expressing different combinations of region-specific transcription factors give rise to distinct types of neurons or glia.

In this respect, it is important to elucidate how the expression of region-specific genes is controlled in neuroepithelial cells. In particular, it has been a salient issue whether the patterns of gene expression and fate choices of neuroepithelial cells are defined by lineage-dependent cell-autonomous mechanisms or environment-dependent non-cell-autonomous regulation (Jessell and Dodd, 1992; Williams and Goldowitz, 1992; Lumsden et al., 1994; Johnson and Tabin, 1995; Simon et al., 1995). Several lines of evidence have suggested an important role for environmental regulation. Transplantation experiments of embryonic brain tissues and/or cells have revealed that neuroepithelial cells are plastic and can change their regional phenotypes dependent on their environments (Nakamura, 1988; Vicario-Abejón et al., 1995). In the chick spinal cord, the specification of dorsoventral identity of neuroepithelial cells is also altered by environmental signals: either removal or implantation of the notochord dramatically altered the dorsoventral expression patterns of *Pax-3* and *Pax-6* (Goulding et al., 1993). In this case, the secreted protein Sonic hedgehog (SHH), also termed Vhh-1 or Hhg-1, or its related proteins have been identified as signals from the notochord that control the restricted expression of these genes (Ekker et al., 1995; Liem et al., 1995). More recent studies have indicated that SHH is also involved in the induction of genes expressed in a subset of cells in the ventral forebrain including *Islet-1* (*Isl-1*), *Nkx-2.1*, *Nkx-2.2* and *Lim-1* (Ericson et al., 1995; Barth and Wilson, 1995; Lumsden and Graham, 1995). It has also been shown that members of the transforming growth factor-β superfamily (BMP-4, BMP-7 and dorsalin-1) are involved in specification of the dorsoventral polarity in the spinal cord by regulating the expression of particular region-specific genes such as *Pax-3* and *Msx1* (Basler et al., 1993; Liem et al., 1995). These studies have emphasized the important role of the environment in defining the expression of region-specific genes.

In contrast, some previous studies have also demonstrated the importance of cell-autonomous mechanisms in maintaining regional identity of neuroepithelial cells. In the chick embryonic hindbrain, a fate-tracing study of single neuroepithelial cells (Lumsden et al., 1994) as well as transplantation studies of particular rhombomere segments (Guthrie et al., 1992; Simon et al., 1995) revealed a role for cell lineage in cell-type determination and *Hox* gene expression. Consistently, the existence of such mechanisms has also been implicated in the forebrain region concerning the expression of the limbic system-associated membrane protein (LAMP) and the PC3.1 antigen (latexin; Arimatsu et al., 1992; Ferri and Levitt, 1993). Furthermore, it has been shown very recently that the expression of the *Otx2*, *Emx2* and *Dlx-1* genes was maintained in neuroepithelial cells cultured in vitro (Robel et al., 1995). These studies have suggested that differential expression of particular antigens and genes in restricted regions of the rodent cerebral cortex was maintained in neuroepithelial cells by certain cell-autonomous mechanisms. However, these studies were carried out using mixtures of cells which are likely to be heterogeneous in terms of the expression of region-specific genes even if they were isolated from restricted areas or domains. Thus, it still remains to be clarified whether cell-autonomy is really operating at the single-cell level in maintaining particular regional identities.

To address these questions, we employed in vitro systems in which the involvement of cell-autonomous mechanisms and environmental signals can be independently manipulated and evaluated. In this study, we first examined the expression of region-specific transcription factors including *Pax-3*, *Pax-5*, *Pax-6*, *Dlx-1*, *Dlx-2*, *Emx2* and *Dbx* in primary culture of neuroepithelial cells from three distinct regions of the developing (E11.5) brain; dorsal forebrain, ventral forebrain and caudal midbrain. Consistent with the results of previous in situ hybridization studies, each of the above genes was indeed differentially expressed in cell populations from distinct regions. We also found that such patterns were maintained when the cells were cultured for 5 days in vitro, suggesting that cell-autonomous mechanisms contribute to this phenomenon. To further explore this possibility, we utilized clonal neuroepithelial cell lines that we recently established from the E11.5 rat forebrain and midbrain (designated as MNS cell lines; Nakafuku and Nakamura, 1995). All of the five cell lines which we characterized shared properties of undifferentiated neuroepithelial cells and could give rise to neurons and glia under particular culture conditions. We demonstrate that each of these cell lines maintained expression of a specific combination of region-specific transcription factors not only during their clonal expansion in vitro, but also when the cells were induced to differentiate. Furthermore, we show that SHH induced different sets of ventral-specific genes in primary culture of distinct regions of the embryonic neuroepithelium as well as in distinct MNS cell lines. These results provide evidence that each neuroepithelial cell harbors some cell-autonomous mechanisms that direct the expression of a particular combination of region-specific genes, and that the same mechanisms also play an important role in regulating how the cell responds to inductive signals from the environment.

MATERIALS AND METHODS

Primary culture of neuroepithelial cells

Neuroepithelia of the forebrain and midbrain were dissected out from E11.5 Sprague-Dawley rats as described previously (Nakafuku and Nakamura, 1995). The day on which the copulatory plug was found was considered as E0.5. Isolation of neuroepithelial cells from specified areas was carried out as follows; the prospective cerebral cortex was cut out from the embryos and used as a dorsal forebrain preparation. From the remaining embryonic head, the ventral forebrain and caudal midbrain regions were dissected out, and surrounding mesenchymal tissues were removed from the primary neuroepithelia. The uppermost position of the optic vesicle and caudal edge of the forebrain vesicle were used as landmarks of the dorsal and caudal margins, respectively, of the ventral forebrain preparations. The rostral margin of the caudal midbrain preparations was defined as half way between the edge of the forebrain vesicle and the rhombencephalic fissure. We noticed that by means of the above method, it was difficult to completely eliminate the most anterior portion of the neural tube, the prospective septal region, from the dorsal forebrain preparation, since no morphological landmark for that region was apparent by inspection at this stage (see Results for details). To further eliminate contaminating non-neural tissues, isolated tissue pieces were incubated at 4°C for 20 minutes in a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 medium (DF; Sanko Junyaku) containing 0.05% (w/v) trypsin and 3 mM sodium ethylenediamine tetraacetic acid (EDTA) as described previously (Murphy et al., 1990). Subsequently, the samples were collected into test tubes and washed three times with DF medium. The preparations were then treated with DF medium containing 0.1% trypsin, 0.001% DNase I (Sigma) and 3 mM EDTA at 37°C for 15 minutes, and single-cell suspensions were made by repeated gentle pipetting. The dissociated cells were divided into two and the first half was directly collected (day 0 preparation). The other half was plated onto poly-D-lysine (10 μg/ml) -coated dishes at densities between 2 to 5×10^5 cells per cm^2 in a standard culture medium consisting of 10% fetal bovine serum (FBS; Sanko Junyaku), 5% horse serum (HS; Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin in DF medium, and was cultured in vitro for 5 days (day 5 preparation). Immunocytochemical studies showed that more than 98% of the isolated cells were positively stained by anti-nestin and the RC1 antibodies, indicating that the preparations were essentially free of non-neural cells. After 5 days in vitro, total cell numbers increased 2- to 3-fold, and the percentage of nestin-positive cells decreased to 65-70%, whereas MAP2-positive neurons and GFAP-positive astrocytes emerged at percentages of 10-20% and 1-5% of the total cells, respectively.

Establishment and maintenance of neuroepithelial cell lines

The neural stem cell lines used in this study, herein designated as MNS (multipotential neural stem) cell lines, were established and maintained as described previously (Nakafuku and Nakamura, 1995). Primary cultured neuroepithelial cells prepared from E11.5 rat forebrain and midbrain were immortalized by infection with the recombinant retrovirus from the Ψ2mycer cell-conditioned medium (Eilers et al., 1989). The retrovirus carries the c-*mycer* gene whereby c-Myc is fused to the ligand-binding domain of the estrogen receptor. With this system it is possible to conditionally activate the c-Myc protein by adding estrogen (e.g. β-E2) to the culture medium (Eilers et al., 1989). Since the retrovirus also carries the *neo*^{r} gene, infected cells were selected with G418. The clonality of the characterized cell lines was confirmed by Southern blot analysis of the provirus integration sites (data not shown). Each cell line was maintained in monolayer culture in the standard medium described above ('monolayer culture'). The medium was changed every 3 days. The detailed procedures to induce differentiation of the cells were described previously (Nakafuku and Nakamura, 1995). Briefly, cells were first allowed to form aggregates in suspension for 3 days in standard medium containing 20 ng/ml bFGF and 1 μM β-E2 ('aggregation culture'). Culture dishes were coated with poly [2-hydroxyethyl methacrylate] (poly HEMA; Sigma) to avoid cell attachment. Cell aggregates were then re-seeded onto poly-D-lysine (100 μg/ml) -coated dishes and were cultured in differentiation medium (standard medium without HS) for 3 or 4 days ('differentiation culture'). The cells cultured under each of the three different conditions were subjected to immunocytochemical studies and RNA preparation.

Expression of SHH and treatment of primary culture and MNS cell lines

CV1 cells stably expressing SHH (CV1SHH) were produced by transfection with a plasmid harboring the full-length chicken SHH cDNA (Ogura et al., 1996). The expression of the N-terminal cleavage product of SHH in CV1SHH cells and in their conditioned media was analyzed using the affinity-purified rabbit anti-SHH antibody (Ab 80) as described by Bumcrot et al. (1995), (a gift from Dr A. McMahon), in which cell pellets were directly lysed in Laemmli's sample buffer, whereas proteins in conditioned medium were concentrated 10-fold by precipitation with 10% trichloroacetic acid. In the case of primary culture of neuroepithelial cells, media conditioned for 48 hours by confluent monolayer of CV1SHH cells and the parental CV1 cells were used for the treatment with SHH and its control, respectively. Preparations of the dorsal and ventral forebrain were isolated and used exactly as described above. For the caudal midbrain, however, its ventral two-third was removed, and the remaining dorsal portion was used as a preparation of the dorsal midbrain. MNS cell lines were treated with SHH by culturing them in contact with a confluent monolayer of CV1SHH cells for 3 days. Control cells were cocultured with the parental CV1 cells in stead of CV1SHH cells. During this 3 day-culture period, no apparent differences in either growth property or viability were observed between the cells in contact with CV1 and those incubated with CV1SHH cells.

Immunostaining

The antibodies used for immunocytochemical studies were described previously in detail (Nakafuku and Nakamura, 1995) with the following exceptions: anti-Pax-6 mouse monoclonal antibody (mmAb; a generous gift from Drs H. Fujisawa and A. Kawakami, Nagoya University; Kawakami et al., unpublished data); anti-MAP2 polyclonal antibody (diluted 1:500; provided by Dr Y. Ihara, University of Tokyo); and the R24 anti-GD3 ganglioside mmAb (LeVine and Goldman, 1988, undiluted conditioned medium of hybridoma, from the ATCC Hybridoma Bank). Indirect immunocytochemical detection of various antigens was performed as previously described (Nakafuku and Nakamura, 1995). A2B5 and R24 antibodies specifically labeled cells in the oligodendrocyte lineage, but not MAP-2-positive neurons or GFAP-positive astrocytes under our experimental conditions. The immunoreactive cells were visualized using fluorescein isothiocyanate (FITC)- or texas red (TR)-conjugated species-specific secondary antibodies (diluted 1:50-100; Cappel or Amersham).

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were prepared from cells or tissues using the acid guanidinium-phenol-chloroform (AGPC) extraction method as described previously (Chomczynski and Sacchi, 1987). RNAs of primary neuroepithelial cells were prepared from day 0 and day 5 cultures (See above for detailed culture protocol). RNAs from MNS cell lines were prepared from three different types of culture: monolayer culture, aggregation culture and differentiation culture (see above). RNA was also prepared from tissues of E11.5 and E15.5 rat forebrain/midbrain

and E11.5 whole embryos deprived of forebrain/midbrain for use as control.

Relative expression levels of various genes in tissue and cultured cell samples were compared by quantitative RT-PCR analysis. cDNAs were synthesized from total RNA by MuMLV reverse transcriptase (Superscript II; Gibco) at 50°C for 1 hour. cDNA derived from 40 ng of total RNA was amplified in a 100 μl PCR reaction containing 5 units of Taq DNA polymerase (Boehringer Mannheim), 1× PCR buffer, 0.4 mM of each dNTP and 100 pmols of each primer (see Table 1) in a thermal cycler (Perkin Elmer, denaturation for 1 minute at 94°C, annealing for 1 minute at 56°C and extension for 2 minutes at 72°C). For *Wnt*-3 and *En-1* transcripts, annealing was carried out at 62°C and 58°C, respectively.

Oligonucleotides used to amplify the cDNAs are listed in Table 1. The identities of the PCR products were confirmed by sequencing the subcloned fragments using an automated sequencer (A.L.F. Sequencer II, Pharmacia and ABI 373A DNA Sequencer, Applied Biosystems). For the *Otx* genes, we used degenerate primers that can detect both *Otx1* and *Otx2*. At the time of this study, the rat sequences were not available for the *Pax-3*, *Pax-5*, *Dlx-1*, *Otx1*, *Emx2*, *Dbx*, *Wnt-3*, *En-1*, *Hox-B1*, *Hox-B3*, or *Nkx-2.2* genes. For these genes, we used the mouse sequences for primer design and compared the similarity of the obtained PCR products with the mouse sequences. More than 97% identity in nucleotide sequence was observed in each clone, which gave sufficient information to distinguish different members of each family. Therefore, we concluded that the obtained cDNA clones were the cognate rat counterparts of the mouse cDNAs.

Relative quantification of gene expression by use of RT-PCR was carried out as follows. During each set of PCR reactions, 8 μl aliquots were collected from the reaction mixtures every 2-3 cycles, and 4 μl of the samples were electrophoresed in 1% agarose or 6% polyacrylamide gels. The gels were then stained with 0.01% SYBR Green I (Amersham) for 45 minutes and fluorescence intensity was measured using FluorImager SI (Molecular Dynamics). For each primer pair, amplified PCR products among different samples were quantified at various cycles within the range of exponential amplification. In control experiments, we confirmed that relative levels of cDNA for a given gene present in different samples could be quantified within the range 20 pg to 10 ng by means of this method (Schneeberger et al., 1995 and Y. N., unpublished data). In all experiments, amplification of β-actin cDNA was carried out alongside, and was used to normalize different cDNA samples. We repeated the above experiments three to five times for each gene using two to five independent preparations of tissue or cell samples, and the normalized mean values (±s.d.) are shown in figures and tables. Cycle numbers used for quantification are also shown in the legends to Figs 2, 4, 7 and 9, and Table 2. To

Table 1. PCR primers used in this study

Gene	Sequence	Reference
β-actin	Sense: 5'-TGC CCA TCT ATG AGG GTT ACG-3' Antisense: 5'-TAG AAG CAT TTG CGG TGC ACG-3'	Rudel et al. 1983
MAP2	Sense: 5'-GAA GGA AAG GCA CCA CAC TG-3' Antisense: 5'-CGT GGC GAT GGT GGT GGG-3'	Kindler et al. 1990
GFAP	Sense: 5'-CAA GCC AGA CCT CAC AGC G-3' Antisense: 5'-GGT GTC CAG GCT GGT TTC TC-3'	Lewis et al. 1984
CNP-II	Sense: 5'-CCG GAG ACA TAG TGC CCG CA-3' Antisense: 5'-AAA GCT GGT CCA GCC GTT CC-3'	Gravel et al. 1994
Pax-3	Sense: 5'-GCT GTC TGT GAT CGG AAC ACT-3' Antisense: 5'-CTC CAG CTT GTT TCC TCC ATC-3'	Goulding et al. 1991
Pax-5	Sense: 5'-GAG CGG GTG TGT GAC AAT GAC-3' Antisense: 5'-CGA GGC CAT GGC TGA ATA CTC-3'	Adams et al. 1992
Pax-6	Sense: 5'-AGT CAC AGC GGA GTG AAT CAG C-3' Antisense: 5'-AGC CAG GTT GCG AAG AAC TCT G-3'	Walther and Gruss 1991
Dlx-1	Sense: 5'-CAA GGC GGG GCA GCT CTG-3' Antisense: 5'-GGG AGA CGG GCA GGA AGC-3'	Price et al. 1991
Dlx-2	Sense: 5'-AGG ATG ACT GGA GTC TTT GAC-3' Antisense: 5'-TCG GAT TTC AGG CTC AAG GTC-3'	Porteus et al. 1992
Otx	Sense: 5'-TAT/C CCI GCI ACI CCI A/CGA/G AAA/G CA-3' Antisense: 5'-ACI AA/GT/C TGT/C TGI CT/GA/G CAT/C TTI GC-3'	Simeone et al. 1992a
Emx2	Sense: 5'-GTC CCA GCT TTT AAG GCT AGA-3' Antisense: 5'-CTT TTG CCT TTT GAA TTT CGT TC-3'	Simeone et al. 1992b
Dbx	Sense: 5'-GCA GA/CG A/GAA A/GGC/G C/GCT GGA GAA-3' Antisense: 5'-TA/GG AA/GT T/GCC GCC AC/TT TCA TC/GC-3'	Lu et al. 1992
Wnt-3	Sense: 5'-GAA GGC TGG AAG TGG GGC GGC-3' Antisense: 5'-ACG CAA TGG CAT TTC TCC TTC CG-3'	Roelink et al. 1990
En-1	Sense: 5'-GAC AGT GGC GGT GGT AGT G-3' Antisense: 5'-GAG GAG CCT GGA GGT GGC-3'	Joyner et al. 1987
Hox-B1	Sense: 5'-CCG GAC CTT CGA CTG GAT G-3' Antisense: 5'-GGT CAG AGG CAT CTC CAG C-3'	Wilkinson et al. 1989a
Hox-B3	Sense: 5'-GTC GAC GCA AAC TGC CAA GC-3' Antisense: 5'-GGG TCA TGG AGT GTA AGG CG-3'	Wilkinson et al. 1989a
Nkx2.1	Sense: 5'-GGC CAT CTC TGT GGG CAG C-3' Antisense: 5'-CTC AGG CGC GTC CCA CAT C-3'	Guazzi et al. 1990
Nkx2.2	Sense: 5'-GGG GGA NCGC AGG CAA GAA G-3' Antisense: 5'-TGT AGG CGG AAA AGG GGA TG-3'	Price et al. 1992
Isl-1	Sense: 5'-GCA GCA TAG GCT TCA GCA AG-3' Antisense: 5'-GTA GCA GGT CCG CAA GGT G-3'	Karlsson et al. 1990

visualize the difference in expression levels among different samples, direct printouts from the fluorescence image analyzer are shown in Figs 4A, 5 and 9C. In these cases, β-actin-normalized cDNA templates were amplified at fixed cycle numbers. For clearer visualization of PCR products, cycle numbers in some cases were 2-3 times larger than those used for quantification, but they were still within the range of exponential amplification (see figure legends for details).

RESULTS

Expression of region-specific genes in neuroepithelial cells in primary culture

To explore the mechanisms that control differential expression of region-specific genes among discrete domains of the forebrain and midbrain, we first examined the expression of a set of transcription factors in neuroepithelial cells cultured in vitro. Neuroepithelia of three distinct regions, including the dorsal forebrain, ventral forebrain and caudal midbrain were dissected out from embryonic rat brain at E11.5 (which roughly corresponds to E9.5-10.5 in mice). Immunocytochemical studies showed that more than 98% of the cells in all the preparations expressed nestin (Lendahl et al., 1990) and RC1 antigen (Edwards et al., 1990), both of which are specific markers for undifferentiated neuroepithelial cells (Fig. 1A-C). From these tissue samples, we isolated RNAs either immediately after dissociation (day of in vitro culture [DIV] 0) or after 5 days in culture (DIV 5), and compared the expression levels of *Pax-3*, *Pax-5*, *Pax-6*, *Dlx-1*, *Dlx-2*, *Emx2* and *Dbx* genes by quantitative RT-PCR analysis (Fig. 2).

We first focused on the three members of the *Pax* gene family, *Pax-3*, *Pax-5* and *Pax-6*, which encode transcription factors related to the *Drosophila* protein paired (Stuart et al., 1993). In situ hybridization studies have shown that *Pax-3*, *Pax-5* and *Pax-6* expressions are first observed by E8.5 in mice along the entire anterior-posterior axis of the neural tube. At later stages, however, their expression domains become restricted to particular areas within the forebrain and midbrain. The rostral limits of *Pax-3* (Goulding et al., 1991) and *Pax-5* (Asano and Gruss, 1992) expression domains retract caudally to dorsal and ventral midbrain, respectively, whereas strong expression of *Pax-6* remains in the dorsal telencephalon at E13.5. In addition, other areas such as ventral thalamus, epithalamus and the ventral midbrain also show significant *Pax-6* expression (Walther and Gruss, 1991). Consistent with these in vivo data, the levels of *Pax-3* and *Pax-5* mRNA were three to twenty times higher in DIV 0 cells of the caudal midbrain preparations than those in the ventral and dorsal forebrain, whereas *Pax-6* was expressed at comparable levels in all of these three regions (Fig. 2).

Next, we examined the expression of three classes of homeobox genes including the *Dlx*, *Emx* and *Dbx* genes, which are vertebrate homologues of the *Drosophila Distal-less* (Boncinelli, 1994; Price, 1993), *empty spiracles*, (Boncinelli et al., 1993) and *H2*, respectively. In the developing mouse brain, *Dlx-1* and *Dlx-2* share most of their expression domains, which reside predominantly in the forebrain, particularly in ventral regions (Bulfone et al., 1993). At E9.5 to 9.75 in mouse embryos, strong expression of *Emx2* is restricted both in the dorsal telencephalon and ventral diencephalon (Simeone et al., 1992b; Shimamura et al., 1995). As development proceeds to E12.5, however, it becomes detectable also in other regions such as the dorsal midbrain (Simeone et al., 1992b). Strong expression of *Dbx* is observed in the midbrain and in some ventral regions of the forebrain, but is scarce in the dorsal forebrain (Lu et al., 1992; N. Takahashi, personal communication). The results of RT-PCR analysis matched the above observations; the levels of *Dlx-1*, *Dlx-2* and *Emx2* expression in the DIV 0 cells from the ventral forebrain were higher (34, 6 and 4 times, respectively) than those from the midbrain, whereas the *Dbx* expression was eight times stronger in the midbrain than in the dorsal forebrain. Comparison of the expression of the above genes between the dorsal and ventral forebrain regions, however, has raised several issues that should be addressed. The expression levels of *Emx-2* in the

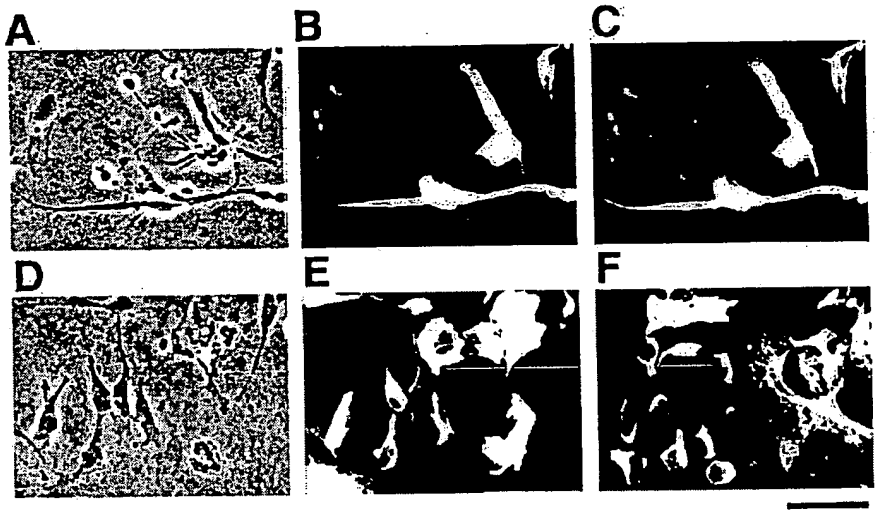

Fig. 1. Indirect immunocytochemical analysis for neuroepithelial cell antigens in primary neuroepithelial cells and MNS cell lines. Primary neuroepithelial cells from E11.5 rat forebrain and midbrain (A-C) and MNS-8 cells (D-F) were stained with antibodies that detect neuroepithelium-expressed antigens. (A) Phase contrast micrograph of primary neuroepithelial cells. The same population of cells was immunoreactive with anti-nestin (B) and the RC1 (C) antibodies. A-C show the same field. In our preparations of neuroepithelia from separate regions, more than 98% of the cells were always nestin/RC1-double positive at DIV 0 (data not shown). (D) Phase contrast micrographs of MNS-8 cells. Fluorescence micrographs showing the expression of nestin (E) and RC1 (F). D and E show the same field. Scale bar, 100 μm.

dorsal forebrain was about twice as high as that in the ventral forebrain, which is generally consistent with the results of previous in situ hybridization studies (Simeone et al., 1992b; Shimamura et al., 1995). In contrast, only three times higher expression of *Dlx-1* and *Dbx* was observed in the ventral than that in the dorsal forebrain, which was smaller than expected from previous studies (Bulfone et al., 1993; Lu et al., 1992). In addition, although the expression of *Dlx-2* is reported to be predominant in the ventral rather than in the dorsal forebrain, it was expressed at similar levels in our preparations of the two regions. One possible reason for these results is that a part of the prospective septal region, which also abundantly expresses *Dlx-1*, *Dlx-2* and *Dbx*, was included in our preparation of the dorsal forebrain. Yet, the overall profiles (also see below) suggest that the results shown here reflect some parts of the differential gene expression patterns between the dorsal and ventral sides of the forebrain.

Next, to test whether the selective expression profiles of the above genes are maintained upon proliferation and differentiation of neuroepithelial cells, we cultured these cells for 5 days in vitro. During this culture period, neuroepithelial cells underwent significant proliferation as well as differentiation into neurons and glia (data not shown), and the cells from each of the three regions maintained gene expression profiles typical of region-specific genes. For example, *Pax-5* expression remained very low in cells derived from the ventral and dorsal forebrain, whereas expression of *Dlx-1* and *Dlx-2* genes remained high in the ventral forebrain. Likewise, both low levels of *Dlx-1* and *Dlx-2* and high levels of *Pax-3*, *Pax-5* and *Dbx* were maintained in cells from the caudal midbrain after in vitro culture. We noticed several exceptions to this general feature: firstly, *Pax-3* was expressed at a significant level in the ventral forebrain, and was even upregulated after 5 days of culture; secondly, *Emx2* and *Dbx* showed marked upregulation in the DIV 5 preparations of caudal midbrain and ventral forebrain, respectively. These observations can be explained by the shift in their expression domains during development. As described above, *Pax-3* expression is initially observed along the entire anterior-posterior axis of the neural tube, and its retraction in the forebrain region occurs between E11- 12 in mice, which corresponds to E13-14 in rats (Goulding et al., 1991). Thus, it appears that our DIV 0 preparation of the ventral forebrain at E11.5 still contained a significant population of *Pax-3*-positive cells. The upregulation of *Emx2* and *Dbx* in DIV 5 cells is also consistent with the extension of their strong expression domains into the midbrain and ventral forebrain at E12.5 in mice as previously described in detail (Simeone et al., 1992b; Lu et al., 1992). In particular, the change in *Emx2* expression from a dorsally enriched to a ventrally enriched pattern after 5 days in culture is consistent with the previous observation that the expression of *Emx2* declines in the dorsal forebrain accompanying the differentiation of cortical neurons, whereas it persists in the ventral regions including hypothalamus even at E17.5 (Simeone et al., 1992b). Thus, we can consider that the expression patterns of the above genes in neuroepithelial cells cultured in vitro reflect, at least in part, their spatial and temporal dynamics observed in vivo.

Taken together, these results raise the possibility that in neuroepithelial cells, certain cell-autonomous mechanisms operate to maintain selective gene expression even after the cells are isolated from the original environment. It is notable that Robel et al. (1995) reported very recently that the expression of *Otx2*, *Emx1* and *Dlx-1* was maintained in telencephalon-derived neuroepithelial cells in vitro, which is consistent with our findings. However, primary cultures inevitably contain heterogeneous

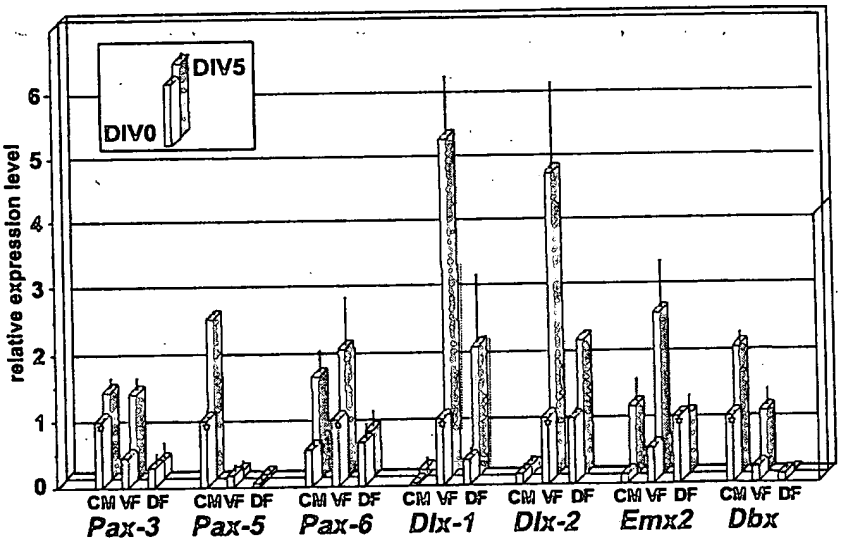

Fig. 2. Analysis of the expression of various region-specific genes in primary cultures of neuroepithelial cells. Quantitative RT-PCR analyses were carried out to examine the expression of the *Pax-3*, *Pax-5*, *Pax-6*, *Dlx-1*, *Dlx-2*, *Emx2* and *Dbx* genes in the primary cultures of neuroepithelial cells from different regions of E11.5 rat brain. Neuroepithelial cells were prepared from the caudal midbrain (CM), ventral forebrain (VF), and dorsal forebrain (DF) as described in Materials and Methods. Total RNA from these cells immediately after dissection (DIV 0; open bars) or after 5 days in vitro (DIV 5; hatched bars) was used for RT-PCR analyses. PCR products were electrophoresed, stained by SYBR Green I, and quantified by measuring the fluorescence intensity. For each primer pair, amplified PCR products among different samples were quantified at various cycles within the range of exponential amplification, and further normalized with the levels of β-actin transcript. For each gene, relative expression levels are shown as mean ± s.d. of three independent reactions, where the highest expression level among the DIV 0 samples from the three brain regions (marked with asterisks) was designated as 1. Essentially identical results were obtained for five independent preparations. Data shown are from one representative preparation. The cycle numbers of PCR reactions used were: 35 for *Pax-3*; 35 for *Pax-5*; 35 for *Pax-6*; 29 for *Dlx-1*; 30 for *Dlx-2*; 35 for *Emx2*; 35 for *Dbx*; and 20 for β-actin.

cell populations expressing different combinations of region-specific genes. Therefore, it is still possible that the maintenance of expression of particular sets of genes is due to non-cell autonomous interactions among cells with different regional identities. Furthermore, we could not examine whether a given single cell in culture continued to express the same sets of genes when it underwent proliferation and/or differentiation. To clarify these points more definitively, it is necessary to analyze clonal and homogeneous cell populations whose proliferation and differentiation can be manipulated in vitro. Therefore, we have established several clonally distinct neuroepithelial cell lines from the E11.5 rat forebrain and midbrain.

MNS cell lines exhibit the properties of multipotential neural stem cells

Detailed characteristics of one of the cell lines used in this study (designated as MNS cell lines) were described previously (Nakafuku and Nakamura, 1995). Here, we show that the established MNS cell lines represent the properties of cultured neuroepithelial cells. We first examined the expression of nestin and the RC1 antigen. MNS-8 cells, like MNS-57 cells that were characterized previously (Nakafuku and Nakamura, 1995), expressed both antigens (Fig. 1D-F). Other clonally distinct cell lines, including MNS-70, -71 and -92, showed similar antigenic phenotypes (data not shown). All these cell lines maintained in monolayer culture continued to express these antigens, indicating that they represent undifferentiated neuroepithelial cells.

We also examined the differentiation potentials of the MNS cell lines. By using the three-step culture protocol (monolayer, aggregation and differentiation culture; described in detail in Materials and Methods), we induced differentiation of the cells, and identified neurons, astrocytes and oligodendrocytes by specific antibodies against microtubule-associated protein 2 (MAP2), glial fibrillary acidic protein (GFAP) and galactocerebroside (GC), respectively. MNS-8 cells, like MNS-57 cells as described previously (Nakafuku and Nakamura, 1995), gave rise to these three cell lineages, whereas MNS-70, -71 and -92 cells generated only MAP2-positive neurons and GFAP-positive astrocytes, but not GC-positive oligodendrocytes under the conditions used in this study (Fig. 3 for MNS-8 and -70; data not shown for the other cells). However, the A2B5 (Gard and Pfeiffer, 1990) and R24 (LeVine and Goldman, 1988) antibodies, which recognize Gq and GD_3 gangliosides on the cell surface, respectively, specifically labeled oligodendrocyte precursor cells with typical unipolar or bipolar morphologies in the differentiated cultures of all the cell lines.

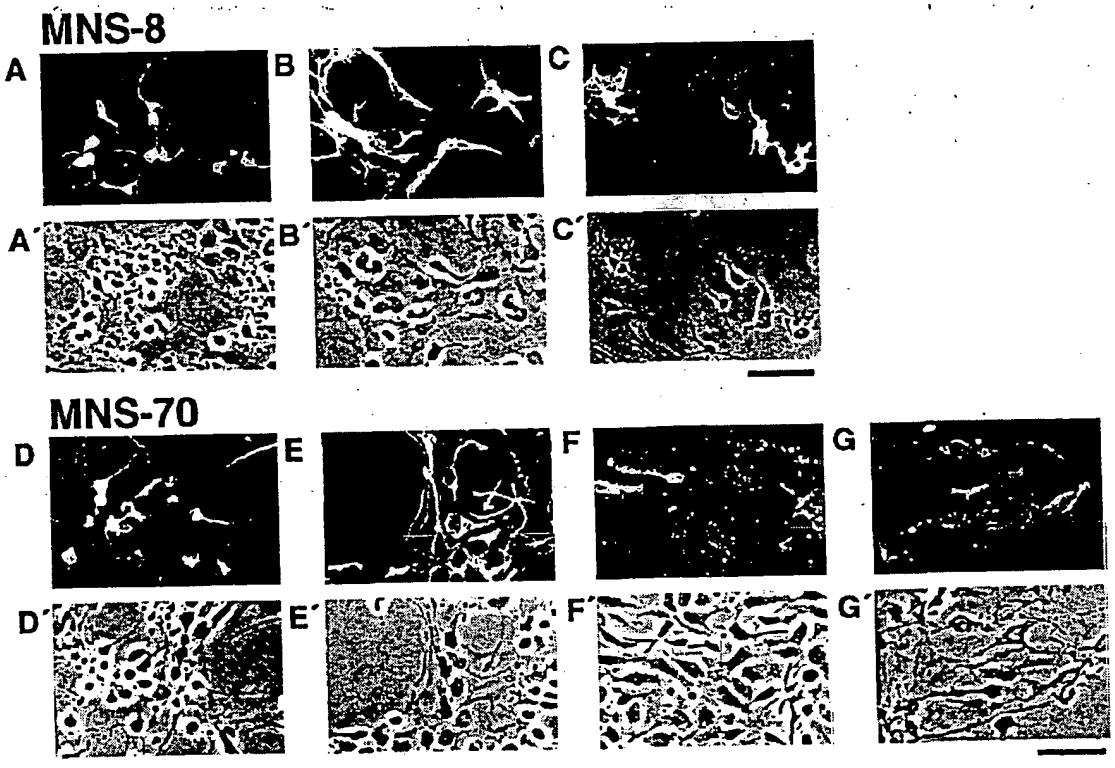

Fig. 3. Generation of both neurons and glia in cultures of MNS cells after induction of differentiation. The cells in 'differentiation culture' (for details, see Materials and Methods) of MNS-8 (A-C) and MNS-70 cells (D-G) were subjected to immunocytochemical analyses using antibodies against various neuron- and glia-specific antigens. Immunofluorescent (A-G) and phase-contrast (A′-G′) images of the same fields are shown side by side. The primary antibodies used were; A and D, anti-MAP2 antibody; B and E, anti-GFAP antibody; C, anti-GC antibody; F, A2B5 antibody; and G, R24 antibody. Scale bar, 50 μm.

Table 2. Expression patterns of various region-specific genes in the five MNS cell lines

	Tissue			MNS cell lines				
	E11.5		E16					
	F/M brain	High expression	F/M brain	8	57	70	71	92
Pax-3	1.00	7.91±0.54 (CM)	0.88±0.14	0.03±0.02	0.03±0.03	0.03±0.02	3.64±0.59	<0.01
Pax-5	1.00	5.93±1.00 (CM)	1.18±0.21	<0.01	0.43±0.07	0.01±0.01	0.01±0.01	0.01±0.01
Pax-6	1.00	7.07±1.33 (VF)	2.32±0.73	0.52±0.08	2.37±0.44	2.40±0.39	0.28±0.11	0.04±0.02
Dlx-1	1.00	17.11±6.53 (VF)	12.88±2.93	0.18±0.03	3.65±1.02	0.82±0.19	8.88±1.96	0.73±0.025
Dlx-2	1.00	7.65±1.38 (VF)	3.23±0.65	0.07±0.04	1.96±1.13	1.46±0.30	3.59±1.32	0.01±0.00
Otx1	1.00	ND	0.85±0.24	0.40±0.10	2.97±0.69	0.20±0.10	0.76±0.20	0.10±0.02
Emx2	1.00	14.4±1.80 (DF)	2.45±0.58	0.41±0.10	1.87±0.35	0.58±0.24	0.13±0.03	1.22±0.32
Dbx	1.00	18.23±2.63 (CM)	1.29±0.46	0.02±0.02	0.64±0.36	8.94±4.46	0.66±0.50	0.05±0.02

In all cases, β-actin was used as an internal control and the expression level of each gene in the control E11.5 forebrain and midbrain tissue sample (F/M brain) was designated as 1.00. All the data shown are mean and s.d. values of three to five independent experiments. The highest level of each gene among the three distinct regions examined in Fig. 2 (indicated in parentheses) are also shown (High Expression). The cycle numbers of PCR used for quantification were: 31 for Pax-3; 35 for Pax-5; 35 for Pax-6; 30 for Dlx-1; 32 for Dlx-2; 33 for Otx1; 32 for Emx2; 35 for Dbx; and 21 for β-actin. DF, dorsal forebrain; VF, ventral forebrain; CM, caudal midbrain; ND, not determined.

In addition to these immunological studies, RT-PCR analyses also demonstrated the expression of lineage-specific genes upon differentiation of the MNS cell lines. Representative results for MNS-8 cells are shown in Fig. 4A and B, in which levels of mRNAs for MAP2, GFAP and 2′,3′-cyclic nucleotide 3′-phosphodiesterase isoform II (CNPII, a marker for oligodendrocytes; Scherer et al., 1994) increased upon induction of differentiation. Based on the above results, we concluded that the MNS cell lines share properties of multipotential neural stem cells present in original preparations of E11.5 forebrain/midbrain neuroepithelium.

The MNS cell lines show distinct expression profiles of region-specific genes

Using these cell lines, we performed intensive analyses of the expression of a series of genes that are restricted to specific domains of the developing forebrain and midbrain, from which all the MNS cell lines originated. First, monolayer culture of each cell line was subjected to quantitative RT-PCR analysis. As shown in Fig. 5A, a high level of *Pax-3* expression was detected only in MNS-71 cells, which was about four times higher than that in the mixture of forebrain and midbrain neuroepithelium and comparable to that in the caudal midbrain-enriched preparations (see Table 2). In contrast, the levels of the *Pax-3* transcript in the other four cell lines were less than 3% of the control. Likewise, a significant level of *Pax-5* expression was detected only in MNS-57 cells. Although *Pax-6* expression was observed in all the cell lines, the levels were highly variable among them. For example, a 60 times higher level of *Pax-6* transcript was detected in MNS-70 than in MNS-92 cells (see Table 2 for results of quantification).

We also examined the expression of four families of the homeobox genes (Fig. 5B). With respect to the *Dlx* family, all the cell lines expressed detectable levels of *Dlx-1* and *Dlx-2* transcripts. However, more than 100-fold differences were observed among different cell lines; MNS-71 cells expressed the highest level (nine times higher than the E11.5 control tissue for *Dlx-1*, and four times for *Dlx-2*), whereas MNS-8

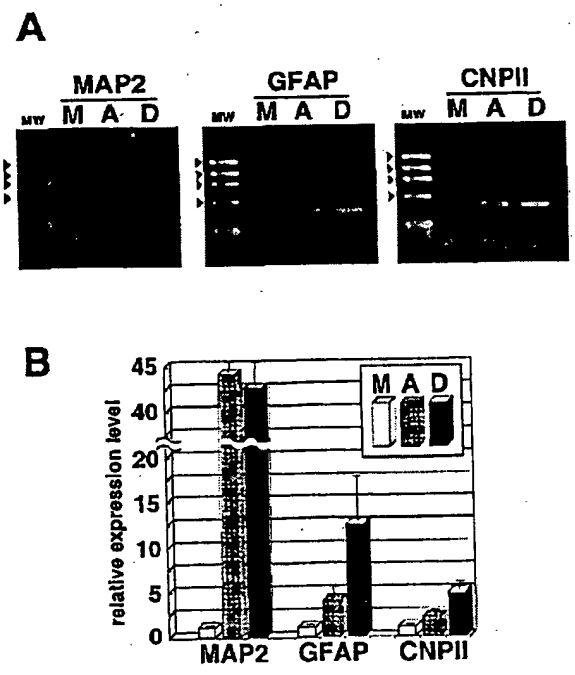

Fig. 4. Induction of neuron- and glia-specific marker genes upon induction of differentiation of MNS cell lines. RT-PCR analysis of the induction of neuronal (MAP2), astroglial (GFAP), and oligodendroglial (CNPII) markers in MNS-8 cells. A shows photographs of PCR products stained with 0.01% SYBR Green I. The positions of molecular size markers (MW) are shown on the left, where arrowheads indicate the ladder of ΦX174 DNA digested with *Hae*III (from the top, 1078 bp, 872 bp, 603 bp, and triplet of 310/281/271 bp). The sizes of specific PCR products are: 405 bp for MAP2; 508 bp for GFAP; and 450 bp for CNPII (see Table 1). Broad bands at the bottom of each figure represent the unincorporated PCR primers. Values for fold-induction of these genes were measured as described in Fig. 2 and are shown in B, where the level in monolayer cells was designated as 1.0. Open bars, monolayer culture (M); hatched bars, aggregation culture (A); and closed bars, differentiation culture (D). The cycle numbers of PCR reactions used for visualization (A) and quantification (B) were: 32 for MAP2; 30 for GFAP; and 32 for CNPII.

cells expressed less than one fifth of the control (Table 2). As shown in Fig. 2, *Dlx-1* and *Dlx-2* transcripts were most abundant in the ventral forebrain, and their levels were 8 to 17 times higher than those in our mixed preparation of E11.5 forebrain and midbrain cells (Table 2). Therefore, levels of *Dlx-1* and *Dlx-2* in MNS-57 and MNS-71 cells are comparable to those in '*Dlx*-high cells' present in the ventral forebrain in vivo. Moreover, it is notable that *Dlx-1* and *Dlx-2* showed parallel expression patterns among different cell lines, which coincides well with their in vivo expression patterns (Bulfone et al., 1993).

Members of the *Otx*, *Emx* and *Dbx* families also showed differential expression patterns among the five cell lines. RT-PCR and sequencing analyses revealed that among the members of these families, expression of only *Otx1*, *Emx2* and *Dbx* could be detected in the MNS cell lines. As shown in Fig. 5B and Table 2, *Otx1* and *Emx2* transcripts were detectable in all the cell lines, but there were marked differences in expression levels. The *Dbx* gene was expressed only in MNS-57, -70 and -71 cells. Again, quantitative comparison with primary tissue samples established that the expression levels of these genes in MNS cell lines were within the range of physiological levels.

Although all the genes described above have restricted expression domains in the developing forebrain and midbrain, some are also expressed in more caudal regions of the neural tube. Thus, we also examined the expression of four other genes that are expressed only in the caudal regions of the brain; *Wnt-3* (Bulfone et al., 1993; Salinas and Nusse, 1992), *En-1* (Davis et al., 1991), *Hox-B1* and *Hox-B3* (Wilkinson et al., 1989a; Lumsden, 1990). Transcripts of these genes were all undetectable in the MNS cell lines (Fig. 5C).

In summary, distinct MNS cell lines expressed different repertoires of region-specific genes, but they were restricted to those found in specific domains of the forebrain and/or midbrain in vivo. Thus, the overall expression patterns are consistent with the forebrain/midbrain origins of the cell lines. Table 2 summarizes the expression profiles of region-specific genes in the five MNS cell lines. It should be noted that each cell line was derived from a single neuroepithelial cell and was clonally expanded. We confirmed that the gene expression pattern observed in each cell line was strictly maintained during repeated passages of the cells. Thus, it is concluded that MNS cell lines possess certain cell-autonomous mechanisms that maintain the expression of specific sets of region-specific genes in vitro even in the absence of environmental signals.

The expression of region-specific genes is maintained during proliferation and differentiation of MNS cell lines

We expected that if cell-autonomous mechanisms are operating independent of environmental signals, a particular set of genes expressed in undifferentiated MNS cells would be maintained even when the cells undergo proliferation and differentiation. To

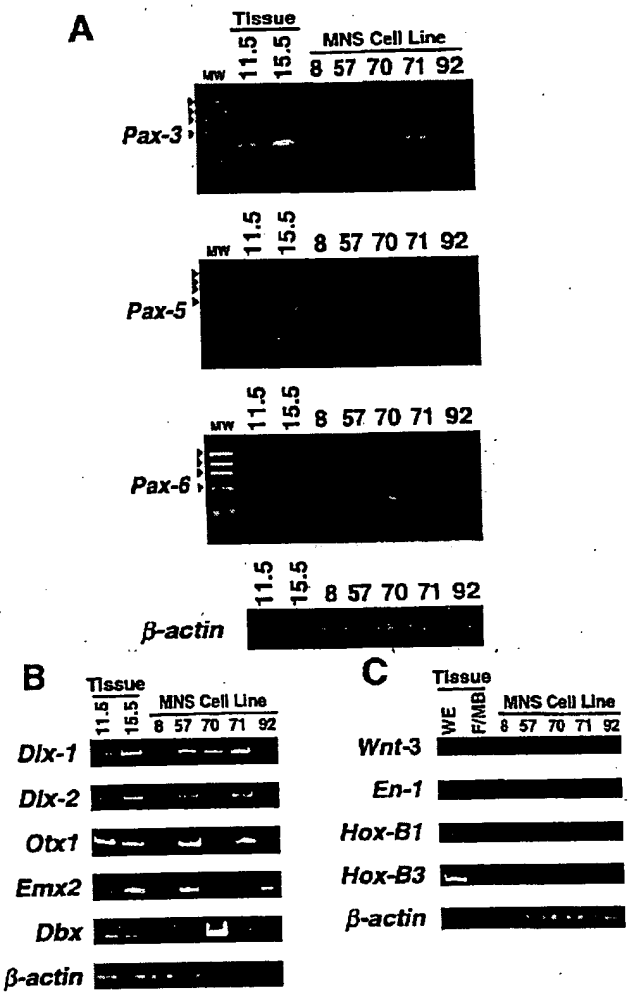

Fig. 5. Differential expression of various region-specific genes in monolayer cultures of MNS cell lines. The levels of expression of the *Pax* genes (A) and the four families of homeobox genes (B) were examined among different MNS cell lines cultured in monolayer as described in detail in Materials and Methods. Expression levels of each gene in the mixed preparation of the forebrain and midbrain tissues from E11.5 and E15.5 embryos are also shown as controls. In panel A, the positions of molecular size markers (MW) are shown by arrowheads on the left as described in Fig 4A. The sizes of specific PCR products are: 418 bp for *Pax-3*; 447 bp for *Pax-5*; and 405 bp for *Pax-6*. Broad bands at the bottom of each figure represent the unincorporated primers. In panel B, weakly stained and faster migrating bands in the lanes for *Dlx-2* show minor nonspecific PCR products, which was confirmed by subcloning and sequencing. See Table 2 for the results of the quantification and PCR cycle numbers used for each gene. In the experiments in Fig. 5, for the clearer visualization of the products, we used cycle numbers 2-3 times larger than those used for quantification. Photographs were obtained directly from the image files generated in the fluorescence image analyzer. The data shown are representative of three to five independent experiments. (C) Results of RT-PCR analyses of *Wnt-3*, *En-1*, *Hox-B1* and *Hox-B3* expression are shown. In all experiments, total RNA from monolayer culture of each cell line was used. The control cDNAs were prepared from E11.5 whole embryos deprived of the forebrain and midbrain (WE) and E11.5 forebrain and midbrain (F/M B), respectively. Note that transcripts of these genes were undetectable in MNS cell lines after 40 cycles of PCR amplification, and their levels were estimated to be at least 100 times lower than those in the control samples.

examine this possibility, we carried out RT-PCR analyses using RNA from cells cultured under three different (monolayer, aggregation and differentiation) conditions. As described above, monolayer culture represents conditions under which undifferentiated MNS cells undergo clonal expansion. In aggregation culture, cell growth was stimulated by the growth factor bFGF and β-E2, an activator of the c-MycER protein, and we assume that during this period the cells undergo commitment to become neurons and glia. In differentiation culture, differentiated neurons and glia were generated, and there were no overall increases in cell number. Representative growth properties of MNS-71 cells are shown in Fig. 6. Similar growth and differentiation patterns were observed in all the MNS cell lines examined in this study (for more detailed description, see Nakafuku and Nakamura, 1995). Furthermore, kinetics of the expression of lineage-specific marker genes clearly showed that differentiation of the MNS cell lines can be conditionally induced (Fig. 4). Thus, this three-step culture protocol allowed us to examine the regulation of region-specific genes in association with proliferation and differentiation of MNS cell lines.

In MNS-71 cells, a high level expression of *Pax-3* remained almost unchanged when proliferation was stimulated in aggregation culture (Fig. 7C). However, its expression level was about three times higher in differentiation culture, where the cells differentiated and ceased proliferation (Figs 6C, 7C). With respect to the *Pax-6* gene, strong (more than 20-fold) induction of expression was observed upon differentiation of MNS-8 cells (Fig. 7A). Weaker but significant upregulation of *Pax-6* was also evident in MNS-70 and -71 cells. Previous studies in mice (Walther and Gruss, 1991) and zebrafish (Macdonald et al., 1994) have revealed that *Pax-6* is expressed in a subset of differentiated neurons. Thus, we asked whether neurons generated from MNS cells express *Pax-6*. As shown in Fig. 8, specific antibodies identified Pax-6 proteins in MAP2-positive neurons in differentiation culture of MNS-70 cells. The expression of two homeobox genes, *Dlx-1* and *Otx1*, was also examined in MNS cell lines. In the three cell lines which we examined, both *Dlx-1* and *Otx1* expression was maintained and their levels increased upon proliferation and differentiation of the cells (Fig. 7). Robel et al. (1995) recently reported that bFGF upregulates the expression of *Otx2* in primary culture of neuroepithelial cells. In our study, proliferation of MNS cell lines were also stimulated by bFGF in aggregation culture, which in many cases, led to the upregulation of the mRNA levels of most of the above genes. Thus, these results may suggest some common regulatory mechanisms for various region-specific genes related to the growth of neuroepithelial cells.

In contrast to the above results, genes that were not expressed in monolayer cultures did not show detectable induction during aggregation or differentiation culture of MNS cell lines. For example, *Pax-3* expression, which was undetectable in monolayer cultures of MNS-8 or -70, was not induced at detectable levels even after aggregation or differentiation (Fig. 7A,B). Similar results were obtained for all the other genes examined in this study (data not shown). Furthermore, it is notable that although the expression of various region-specific genes examined were all upregulated upon induction of differentiation, the overall expression profile in monolayer culture of each cell line was conserved in this process. For example, in MNS-71 cells, high levels of expression of *Pax-3* and *Dlx-1* were maintained among three culture conditions, while the levels of *Pax-6* and *Otx1*, which were relatively lower than

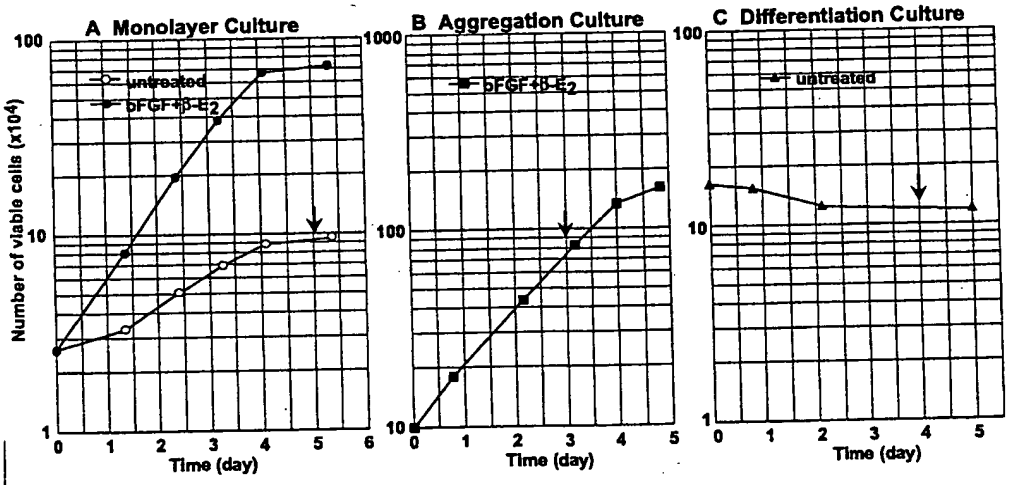

Fig. 6. Growth properties of MNS-71 cells under different culture conditions. MNS-71 cells were cultured under the three different conditions (monolayer, aggregation, and differentiation culture, for details, see Materials and Methods), and the growth of the cells was examined. For the monolayer culture (panel A), the cells were seeded at a density of 1×10^4 cells per ml in DF medium containing 10% FBS and 5% HS. Twenty-four hours later, the medium was replaced by the above medium supplemented with (closed circles) or without (open circles) 20 ng/ml bFGF and 1 μM β-E_2, and the subsequent increase in cell number was monitored daily. For the aggregation culture (B), cell suspensions at a density of 1×10^5 cells per ml were seeded onto poly HEMA-coated dishes in the above medium plus bFGF and β-E_2. For the differentiation culture (C), the cells aggregated in the presence of bFGF and β-E_2 for 3 days were re-seeded onto poly-D-lysine-coated dishes, and incubation was continued in DF medium plus 10% FBS without bFGF or β-E_2. The arrow in each panel indicates the time when the cells were harvested and subjected to RNA preparation.

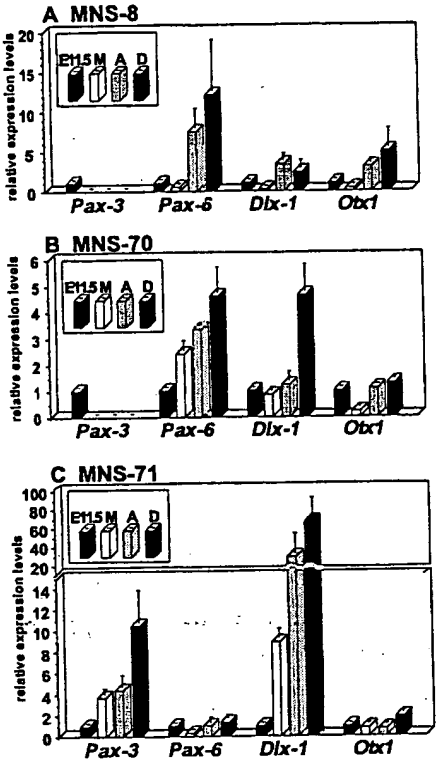

Fig. 7. Regulation of *Pax-3*, *Pax-6*, *Dlx-1* and *Otx1* expression upon differentiation of MNS cell lines. MNS cell lines were cultured under three different conditions as described in Fig. 3. Total RNAs were isolated, and relative expression levels of region-specific genes in MNS-8 (A), MNS-70 (B) and MNS-71 (C) were quantified as described in Fig. 2. In each set of genes and cell lines, the expression level in the forebrain and midbrain neuroepithelia from E11.5 embryos (E11.5; solid bar) was designated as 1.0, and those in monolayer (M, open bars), aggregation (A, light shaded bars) and differentiation (D, dark shaded bars) cultures were shown as mean (±s.d., n=3-5) values. The cycle numbers used for quantification were the same as those described in Table 2.

those in other cells, remained low under different culture conditions. Likewise, MNS-70 maintained the pattern of high expression of *Pax-6* and *Dlx-1* and low expression of *Pax-3* and *Otx1* under all culture conditions. This general feature is consistent with the data obtained in primary culture of neuroepithelial cells shown in Fig. 2, in which undifferentiated neuroepithelial cells at DIV 0 underwent significant proliferation and differentiation during 5 days in culture. Thus, the most important conclusion from these results is that the differential expression patterns of region-specific genes in undifferentiated MNS cell lines are maintained even after induction of their proliferation and differentiation.

SHH induces differential expression of ventral marker genes in MNS cell lines

We next asked whether distinct cell-autonomous properties among different neuroepithelial cells influence the responsiveness to environmental signals. For this purpose, we examined their responses to SHH, which is one of the best characterized signaling molecules involved in regional specification. It has been shown that SHH induces the expression of a series of ventral cell-specific transcription factors including *HNF-3β*, *Isl-1*, *Nkx-2.1*, *Nkx-2.2* and *Lim-1*, thereby playing a crucial role in specification of the ventral phenotype of neuroepithelial cells (Echelard et al., 1993; Roelink et al., 1994; Martí et al., 1995; Ericson et al., 1995; Barth and Wilson, 1995). In this study, we utilized CV1 cells which express chicken SHH at high levels and secrete its N-terminal cleavage product in the conditioned medium (Ogura et al., 1996; Fig. 9A). We first isolated primary cultures of neuroepithelial cells from three distinct regions, and compared their expression levels of the *Isl-1*, *Nkx-2.1* and *Nkx-2.2* genes (Fig. 9B). Consistent with previous in situ studies in chick and mouse embryos (Ericson et al., 1995; Shimamura et al., 1995), *Nkx-2.1* expression was restricted in the ventral forebrain, and its levels in the dorsal forebrain and midbrain were less than 0.3% and 0.1% of that in the ventral forebrain, respectively. The expression of *Nkx-2.2* and *Isl-1* was detected both in ventral forebrain and midbrain preparations, but they were much weaker in the dorsal forebrain (1.3% and 4.5 % of that in the ventral forebrain for *Nkx-2.2* and *Isl-1*, respectively). Upon incubation with the medium conditioned by CV1SHH cells, the levels of the all

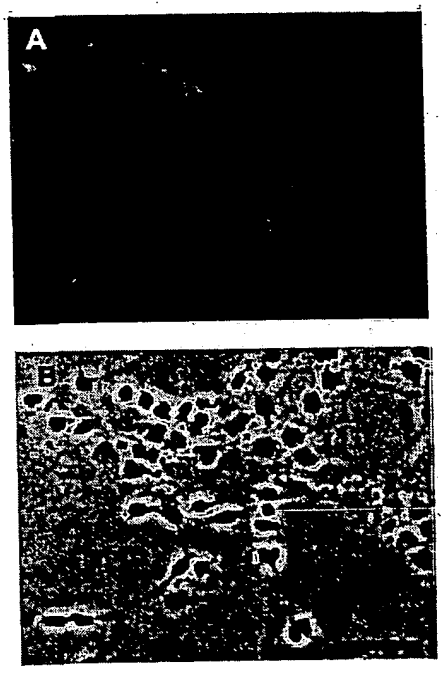

Fig. 8. The Pax-6 protein was expressed in MAP2-positive neurons generated from MNS-70. MNS-70 cells grown under differentiation culture conditions were analyzed by double immunostaining with anti-Pax-6 and anti-MAP2 antibodies. Secondary antibodies used were FITC-conjugated anti-mouse IgG for Pax-6 and TR-conjugated anti-rabbit IgG for MAP2. (A) Fluorescence micrograph showing the coexpression of Pax-6 (green) and MAP2 (red). Cells that expressed both proteins are shown in yellow. (B) Phase contrast micrograph showing the same field as A. Scale bar, 100 μm.

three genes in the dorsal forebrain was dramatically elevated and were close to those of the ventral forebrain (Fig. 9B). These results are consistent with the idea that SHH has the ability to ventralize the dorsal neuroepithelium (Ericson et al., 1995; Lumsden and Graham, 1995). In the ventral forebrain, which endogenously expressed much higher levels of *Nkx-2.2* and *Isl-1*, they remained almost unchanged after the treatment with exogenous SHH. This is probably due to the presence of large numbers of SHH-expressing cells in this preparation. Dorsal midbrain cells expressed *Nkx-2.1* and *Nkx-2.2* at very low levels, and exogenous SHH did not increased the levels of either genes significantly. In particular, the absence of *Nkx-2.1* induction in the midbrain and its remarkable induction in the dorsal forebrain coincided with its forebrain-specific expression in vivo (Shimamura et al., 1995). It is also consistent with a previous report describing that SHH could induce *Nkx-2.1*-positive cells in explant culture of the forebrain neuroepithelium but not of the hindbrain (Ericson et al., 1995). These results indicate that neuroepithelial cells in distinct regions possess distinct properties in terms of the expression of ventral-specific genes and their responsiveness to SHH.

Next we asked how clonal MNS cell lines respond to SHH. Among the monolayer cells of MNS-8, -57, -70, -71 and -92, *Isl-1* was expressed only in the MNS-71 cells (the relative level of expression was 0.77±0.39, where that of the control E11.5 forebrain/midbrain tissue was designated as 1.00, n=3), and *Nkx-2.2* was expressed only in MNS-71 cells (0.33±0.08, n=3; also see Fig. 9C). In all the other cases, expression levels of *Isl-1*, *Nkx-2.1* and *Nkx-2.2* were less than 5% of the control tissue. Furthermore, none of the cell lines expressed *HNF-3β*, *Lim-1*, or *SHH* at a detectable level (data not shown). When MNS cell lines were grown in contact with CV1SHH cells, they showed differential induction profiles of the above genes (Fig. 9C). Upon contact with the CV1SHH cells, MNS-70 cells exhibited clearly elevated (more than 50- to 100-fold) levels of expression of *Isl-1*, *Nkx-2.1* and *Nkx-2.2*, which were comparable to those detected in the E11.5 forebrain/midbrain neuroepithelium (0.39±0.06 for *Isl-1*, 0.58±0.14 for *Nkx-2.1* and 2.03±0.45 for *Nkx-2.2*, n=3), although the accurate values for fold-induction were uncertain because of the very low levels of their expression in the control (SHH-untreated) cells. MNS-71 cells showed a very weak but detectable induction of *Nkx-2.1* in response to SHH, but the expression of *Isl-1* and *Nkx-2.2*

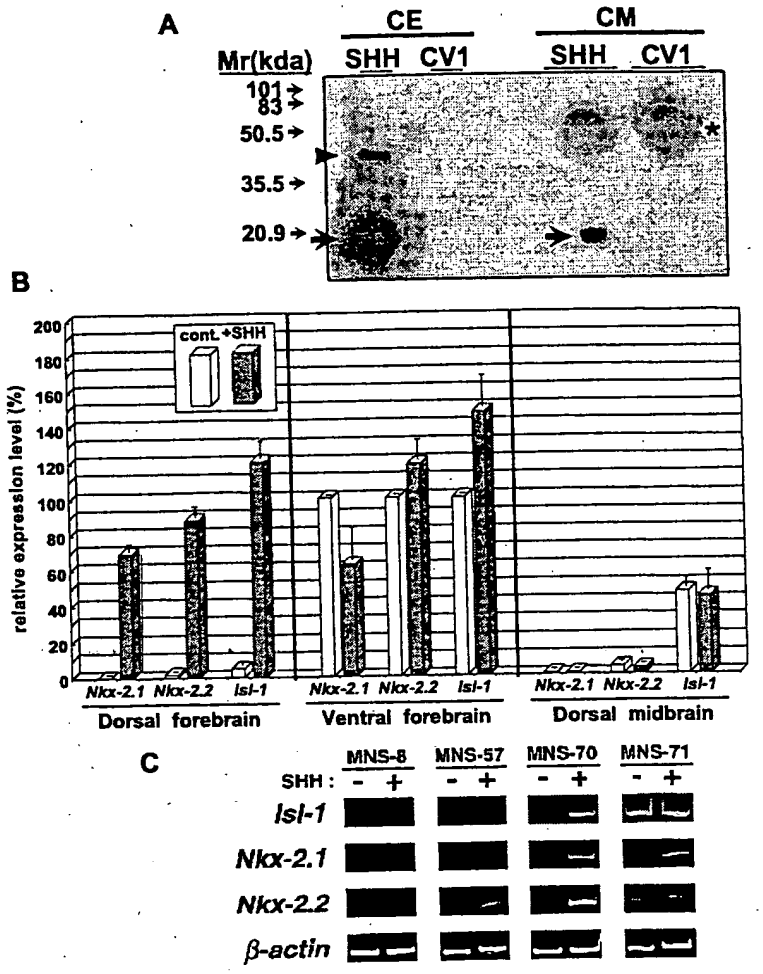

Fig. 9. SHH induced expression of ventral neuroepithelium-specific genes in MNS cell lines. (A) Western blot analysis of SHH expressed by CV1SHH cells. Whole cell extracts (CE) and conditioned media (CM) were prepared from 5×10^3 cells of the control and CV1SHH. These samples were subjected to 15% polyacrylamide gel electrophoresis and blotted with the anti-SHH antibody. The arrowhead and arrows with apparent relative molecular masses of 46×10^3 and 20×10^3 correspond to the full-length and its amino-terminal cleavage products of chicken SHH, respectively. The nonspecific bands in lanes of CM (marked by an asterisk) were due to the presence of large amounts of bovine serum albumin in the culture medium. (B) Primary culture of neuroepithelial cells was established as described in Materials and Methods, in which cells of three distinct regions, the dorsal forebrain, ventral forebrain, and dorsal midbrain were isolated separately. Note that in these experiments, the ventral two-thirds was removed from the caudal midbrain preparation used in Fig. 2. Subsequently, the cells were treated with the medium conditioned by the control CV1 (cont., shown by open bars) or CV1SHH (+SHH, shaded bars) cells for 3 days, and RNA was prepared for quantitative RT-PCR analysis of the *Isl-1*, *Nkx-2.1* and *Nkx-2.2* genes. For each gene, relative expression levels are shown as mean ±s.d. of three independent experiments, where the expression level in the ventral forebrain was designated as 100. (C) RNAs were prepared from the MNS cell lines cultured for 3 days in contact with control (–) or CV1SHH (+) cells. The induction of *Isl-1*, *Nkx-2.1* and *Nkx-2.2* was analyzed by quantitative RT-PCR. The cycle numbers of PCR reactions for Panels B and C were: 29 for *Isl-1*; 35 for *Nkx-2.1*; 32 for *Nkx-2.2*; and 20 for β-actin.

remained almost unchanged (0.69±0.15 for *Isl-1*, and 0.57±0.26 for *Nkx-2.2*, n=3, where the levels in the control cells were designated as 1.00). MNS-57 cells underwent significant induction of the *Nkx-2.2* expression (the level in the SHH-treated cells was 0.26±0.12 compared with 1.00 of the E11.5 sample, n=3) but not of *Isl-1* and *Nkx-2.1*, whereas none of these genes were induced in MNS-8 cells. In contrast, we did not observe detectable induction of *HNF-3β* or *Lim-1* in any of these cell lines (data not shown). Similar differential induction patterns were obtained by using the conditioned medium from CV1SHH cells but not with that from the parental CV1 cells (data not shown), indicating that the induction of the ventral genes directly resulted from exposure to SHH. These results demonstrate that distinct MNS cell lines, which maintain the expression of different sets of region-specific genes in vitro, show specific and differential responsiveness to the same inductive signal from the environment, i.e. SHH.

DISCUSSION

The generation of neural cell diversity in the developing central nervous system is thought to be regulated by both cell-intrinsic and -extrinsic mechanisms (see Jessell and Dodd, 1992; Ruiz i Altaba, 1994; Simon et al., 1995; Lumsden and Graham, 1995 for discussion). However, it is not fully understood how these two mechanisms contribute to the determination of a particular fate of each progenitor cell. Recently, a number of molecules have been identified that potentially regulate this complex process. These genes, collectively called region-specific genes, are expressed in the developing neuroepithelium in a spatially and temporally restricted manner, and have been implicated in the specification of particular domains or layers of the brain. Thus, studies on the regulation of these genes would provide crucial information to facilitate understanding of the molecular nature of the above two mechanisms. Recent studies have uncovered the important roles of inductive signals from the environment for the regulated expression of region-specific genes (Jessell and Dodd, 1992; Johnson and Tabin, 1995), but little is known about the involvement of the cell-autonomous mechanisms. In this study, we have established in vitro culture systems which enable us to study how cell-autonomous mechanisms and environmental signals contribute to the regulation of region-specific genes in neuroepithelial cells.

First, we showed that neuroepithelial cells from distinct regions of the brain express region-specific genes, including *Pax-3*, *Pax-5*, *Pax-6*, *Dlx-1*, *Dlx-2*, *Emx2* and *Dbx* at different levels. The overall pattern in each preparation was generally consistent with the results of previous in situ hybridization studies (Fig. 2). In addition, when cultured for 5 days free from the influences of cells in other regions, cell populations did not undergo significant changes in the overall gene expression pattern. These results support the notion that certain cell-autonomous mechanisms play an important role in maintaining the expression of particular sets of genes in neuroepithelial cells.

Next, we demonstrated that differential expression of region-specific transcription factors among different neuroepithelial cells can be reproduced in immortalized cell lines. MNS cell lines used in this study, which were established from E11.5 rat forebrain and midbrain (Nakafuku and Nakamura, 1995), shared properties of neural stem cells in that they expressed neuroepithelium-specific antigens and could generate neurons and glia under particular culture conditions (Figs 3, 4). Since MNS cell lines are derived from clonally distinct cells, they provide a useful model system in which to examine what types of region-specific genes are expressed in single neuroepithelial cells, and to study how they are regulated under conditions in which the influence of environmental signals and cell-cell interactions among heterogenous cell populations can be eliminated. We found that five distinct MNS cell lines expressed different combinations of transcription factors expressed in restricted regions of forebrain and midbrain. In contrast, region-specific genes that are expressed only in the caudal brain, including *Wnt-3*, *En-1*, *Hox-B1* and *Hox-B3*, were all below detectable levels in these cell lines. These results are consistent with the forebrain/midbrain origin of the MNS cell lines, and it is unlikely that the observed gene expression profiles of the MNS cell lines have resulted from some random events related to immortalization or in vitro culture. Furthermore, we demonstrated that these expression profiles remained essentially unchanged upon proliferation and differentiation (see Fig. 7). Immunocytochemical analysis demonstrated that when MNS-70 cells, which expressed the highest level of *Pax-6* among the cell lines examined, were induced to differentiate, *Pax-6*-positive neurons were indeed generated (Fig. 8). These results strongly suggest that at least in some cases, expression of region-specific genes in undifferentiated neural stem cells is directly inherited to their neuronal (and possibly also glial) progeny.

In relation to the above results, comparison of our data shown in Fig. 2 and Table 2 with the available information from a number of previous in situ hybridization studies (Bulfone et al., 1992; Puelles and Rubenstein, 1993; Stoykova and Gruss, 1994; Rubenstein et al., 1994 for details) demonstrated that the overall gene expression patterns found in some of the MNS cell lines closely matched those in particular regions of the developing brain. For example, MNS-70 cells expressed significant levels of *Pax-6*, *Dlx-1*, *Dlx-2* and *Dbx*, but not *Pax-3* or *Pax-5*. This profile is reminiscent of that in the septal region of the forebrain. However, MNS-8 cells shared similar expression patterns with the dorsal telencephalic region shown in Fig. 2 in that they expressed *Pax-6*, *Otx1* and *Emx2* at significant levels but not *Dlx-1*, *Dbx*, *Pax-3*, or *Pax-5*. MNS-57 cells expressed all the genes examined except *Pax-3*, but the relatively high levels of *Dlx-1*, *Dlx-2* and *Emx2* suggest its ventral forebrain origin, although our data shown in Fig. 2 did not clearly distinguished between the ventral and dorsal forebrain. We consider that these results may suggest the intriguing possibility that each MNS cell line inherited a particular regional identity from the neuroepithelial cell from which it originated. It should be noted, however, that although our data in Fig. 2 clearly showed differential expression profiles of various region-specific genes among distinct brain regions in vivo, we still do not know their exact expression patterns in particular single cells in a given region. For example, it is possible that even in the ventral forebrain where *Dlx-1* is strongly expressed, some cells express it at only very low levels. Thus, the comparison of the combinations of expressed genes alone is not enough at present to definitively

assign the position where each cell line was derived. Nevertheless, strict maintenance of gene expression profiles in MNS cell lines upon continuous cell growth and differentiation makes it likely that a particular set of genes expressed in individual cell lines reflect their distinct origins.

Based on the above results, we can conclude that certain cell-autonomous mechanisms play important roles in maintaining the expression of a specific set of region-specific genes. Several previous studies have also implicated similar mechanisms for the establishment of regional identity in the developing brain. For example, the limbic system-associated membrane protein (LAMP) is specifically expressed in the limbic cortex but not in other cortical areas (Horton and Levitt, 1988). Transplantation and in vitro culture experiments have shown that its specific expression in the limbic cortex is maintained after isolation from the original environment (Barbe and Levitt, 1991; Ferri and Levitt, 1993). Differential expression of the PC3.1 antigen (latexin), which also revealed regional heterogeneity of the developing cerebral cortex in vivo, was conserved in cultured neuroepithelial cells (Arimatsu et al., 1992). Likewise, chick/quail heterotopic transplantation demonstrated that midbrain and hindbrain neuroepithelia maintained their predetermined fates even after being placed into other brain regions (Nakamura, 1990). These studies collectively support our conclusion that cell-intrinsic mechanisms indeed play important roles in determination of the regional fate of neuroepithelial cells.

Finally, we examined the possible cooperative actions of the cell-autonomous and non-cell autonomous mechanisms in the regulation of region-specific gene expression. We first showed that SHH, which is one of the best characterized inductive signals from the environment (Martí et al., 1995; Roelink et al., 1995; Hynes et al., 1995; Ericson et al., 1995; Barth and Wilson, 1995), induced the expression of various ventral-specific genes, including *Nkx-2.1*, *Nkx-2.2* and *Isl-1* in primary cultures of distinct regions of the embryonic neuroepithelium (Fig. 9B). The dorsal forebrain expressed high levels of all these genes in response to SHH, and its pattern resembled that in the ventral forebrain. This result supports the notion that SHH acts as a major ventralizing signal in the forebrain region. A clear difference between the forebrain and midbrain was evident in that the dorsal midbrain did not express *Nkx-2.1* with or without exogenous SHH. These results were consistent with previous studies showing that neuroepithelial explants, derived from distinct regions, generated different cell types in response to SHH. In spinal cord explants SHH induced *Isl-1*-positive cells with the identity of motoneurons (Martí et al., 1995; Roelink et al., 1995), whereas it generated dopaminergic neurons in midbrain-derived explants (Hynes et al., 1995). On the other hand, SHH specifically induced *Nkx-2.1* and *Lim-1* in a population of forebrain-derived neuroepithelial cells in chick embryos (Ericson et al., 1995). However, as in the case of our study using primary culture of neuroepithelial cells, these studies utilized explant cultures which contained heterogeneous cell populations, and hence the contribution of cell-cell interactions among these populations in each explant has remained unclarified. Furthermore, distinct responsiveness among neuroepithelial cells has not yet been fully characterized at the single-cell level. To address this point, we extended the above observations by examining the properties of MNS cell lines. We demonstrated that the cell lines had the ability to respond to SHH and expressed these ventral cell-specific genes (Fig. 9C). Furthermore, we found that the combinations of the induced genes differed among different cell lines. These results strongly suggest that cell-intrinsic properties of neuroepithelial cells indeed define the responsiveness to environmental signals, as well as the repertoires of genes expressed in their absence. It is notable that SHH induced *Nkx-2.1* expression in MNS-70 and MNS-71 cells, which is consistent with their forebrain origin as suggested by the expression patterns of other region-specific genes (discussed above), since *Nkx-2.1* was specifically induced in the forebrain as shown in Fig. 9B. In addition, the use of homogeneous cell populations enabled us to clearly conclude that intrinsic properties of the cells themselves but not cell-cell interactions among heterogenous populations are responsible for specifying how neuroepithelial cells respond to SHH.

In summary, it is highly likely that both cell-autonomous mechanisms and environmental factors contribute cooperatively to the differential and regulated expression of the genes specifying the identities of neuroepithelial cells. Many questions, however, still remain to be answered. At present, we do not know exactly how particular region-specific genes are activated in each cell line, how they are maintained, or how various environmental signals contribute to the initial activation and subsequent modulation of region-specific genes. We propose that the MNS cell lines described here will serve as a useful in vitro model to clarify the above questions at the molecular level. Such studies would provide further insight into the molecular basis of regional specification in the developing brain.

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EXHIBIT E

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Interactive report

Regional specification of rodent and human neurospheres

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Abstract

Neural precursor cells were isolated from various regions of the developing rat and human brain and grown in culture as aggregates termed neurospheres. We asked whether cells within human and rodent neurospheres are identical, or whether they have species specific characteristics or differences based on their region of origin. Under our culture conditions, rodent neurospheres isolated from the cortex (^{ctx}NS) and striatum (^{str}NS) grew faster than those from the mesencephalon (^{mes}NS), but stopped growing after only eight to ten population doublings. In contrast, human neurospheres under identical culture conditions, continued to grow for over 40 population doublings. Following migration and differentiation of both rodent and human cultures, ^{ctx}NS and ^{str}NS generated high numbers of small neurons whereas ^{mes}NS generated small numbers of large neurons with many long fibres. Only very rare neurons from ^{mes}NS expressed dopaminergic markers, and thus may require further signals to fully mature. While the rat neurospheres generated high numbers of oligodendrocytes, very few were found to develop from human neurospheres from any region after a few weeks of passaging. FACS analysis revealed a unique population of smaller cells within human ^{str}NS and ^{ctx}NS, which appeared to be neuronal progenitors. However, large cells within neurospheres were capable of generating these small neuronal progenitors following further proliferation. Together, our data show that rat and human neurospheres have unique characteristics with regard to growth and differentiation, and that the majority of precursor cells within neurospheres are regionally specified to generate set numbers of neurons. These findings have important implications for understanding the nature of proliferating neural precursors isolated from the developing CNS, and their potential for brain repair.

1. Introduction

Small populations of stem cells exist in the developing and adult rodent brain, which can generate progenitor cells capable of differentiating into neurons, astrocytes or oligodendrocytes [17]. However, fundamental challenges within this field of biology are (i) to establish how cell-autonomous programs interact with environmental signals to direct the phenotypic fate of these cells and (ii) to understand the mechanisms underlying their self-renewal capacity [2]. One technique for growing cells derived from the germinal zones of either the developing or adult CNS involves the generation of free floating spherical aggregates termed 'neurospheres'. This method was developed for rodent tissues a number of years ago [32] and has recently been adapted for the long-term growth of human neurospheres by ourselves and others [10,45,49]. Neurospheres from rodents consist of both multipotent stem cells and more restricted progenitors [33] and, as such, are considered to comprise a heterogeneous population of neural precursor cells (NPCs) [41]. Although the selection of sphere forming cells from primary neurogenic zones within differentiating CNS regions of the animal is possible [34,47], the reliable distinction between true stem cells and more restricted progenitors within expanded populations of neurospheres has been limited by the lack of available cell-type specific markers. It is possible that such cells may be regionally specified. If this were true, neurospheres generated from different brain regions would retain some features of this region, even following expansion in culture. Alternatively, is it possible that a common

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stem cell exists along the entire extent of the neuroaxis which, following isolation, would behave in a similar fashion irrespective of its origin. In addition to these regional complexities, there are likely to be differences between species with regard to general stem cell biology that need to be addressed, particularly in the context of potential clinical applications.

Clearly, tissues derived from one brain region and expanded in culture can take on the phenotype of another following transplantation [16,36,40] or, in more extreme cases, can trans-differentiate into cells of different dermal origin when injected into irradiated mice or blastocysts [5,12]. These results suggest that at least some neuroepithelial cells are extremely plastic and environmentally specified, with very little evidence of genetic determination. However, these types of cells may be only a very small fraction of cells within such cultures. Although cells derived from different embryonic brain regions and expanded in culture adopt host region phenotypes when transplanted, there is evidence that some of these cells retain a molecular memory of where they came from, based on the expression of regionally expressed genes [28,35,53] or proteins [14]. In addition, although cells derived from the embryonic forebrain migrate and differentiate within hind-brain regions when transplanted into neonates [9], these cells continue to express markers associated with their region of origin [27]. Very recently, the region specific differentiation of spinal cord progenitor cells [52] and mammalian neural crest cells [51] has also been reported following transplantation. Thus, cell autonomous mechanisms may exist to control the fate of these cells. Neurospheres generated from the human fetal brain produce large number of neurons [42], but those from the spinal cord exclusively produce astrocytes using slightly different growth conditions [3,31], suggesting that some regional specification also exists along the human neuroaxis. However, a direct comparison of proliferative and phenotypic potential of neurospheres generated from different brain regions and grown under identical culture conditions, has not previously been undertaken.

To address these issues, we compared the growth and differentiation of non-genetically modified epidermal growth factor (EGF)- and fibroblast growth factor-2 (FGF-2)-responsive neural precursors isolated from various regions of the embryonic rat and human brain.

2. Materials and methods

2.1. Rodent neural precursor cell cultures and proliferation studies

The cortex, striatum (comprising both medial and lateral ganglionic eminences) and ventral mesencephalon (VM) were dissected from embryonic day 14 (E14) rat brain. Human embryonic tissue (between 6 and 21 weeks post conception) was collected following routine terminations of pregnancy. The methods of human tissue collection conformed with the arrangements recommended by the Polkinghorne Committee for the collection of such tissues and to the guidelines set out by the United Kingdom Department of Health. The same regions plus the cerebellum and thalamus were isolated from human fetal samples. Tissue was treated with trypsin (0.1% for 20 min), washed in DMEM and then dissociated into a single cell suspension. Cells were initially seeded at a density of 400,000 per ml into T75 flasks containing 20 ml of defined serum-free medium (DMEM:HAMS-F12 at 3:1) supplemented with B27 (2% v/v), epidermal growth factor (EGF, 20 ng per ml) and fibroblast growth factor (FGF-2, 20 ng per ml) with heparin (5 μg per ml).

Cells from all regions of both the rat and human tissue formed neurosphere cultures during the first 2–5 days of growth. Due to differences in expansion rates between the rat and human cells, they were then passaged differently from this point. The rat cultures were passaged at 7 days (P1) and then every 14 days (P2, P3) by chopping spheres into 200-μm sections, which were then re-seeded into fresh growth medium containing both EGF and FGF-2 and B27 at a density equivalent to ~200,000 cells per ml. The sectioning of neurospheres has previously been developed as a method for optimising the in vitro expansion of human NPCs [45]. Estimates of rat cell expansion were undertaken every 7 days by the removal of aliquots of cells from the flasks, which were then dissociated and counted using the trypan blue exclusion method. To establish a relationship between cell number and neurosphere volume, sequential measurements of sphere size were undertaken for individual rat neurospheres between 7 and 16 days in culture.

The human neurospheres were passaged every 14 days by sectioning of spheres into 350-μm sections, that were re-seeded into fresh growth medium at a density equivalent to 200,000 cells per ml. Half the growth medium was replenished every 4th day. Passaging of cells was undertaken every 14 days. After the first passage all cells were grown in EGF and FGF-2 supplemented media, but B27 was replaced with the supplement N2 (1% v/v, Gibco). At 4 weeks of growth all cultures were switched to EGF alone and fed every 4 days and passaged every 14 days thereafter. These slight differences to the rodent cultures were introduced because the human cultures could be expanded for long periods of time without the addition of either B27 or FGF-2 and these factors were not required for continual growth of the human cells for up to 250 days [45].

2.2. In vitro differentiation studies and neuronal quantification

We used a cell migration assay, which has been previously described in detail [7], to assess the differentiation potential of neurospheres grown from either the rat or

human. Whole neurospheres generated from the different CNS regions and at sequential passages were plated directly onto poly-L-lysine/laminin-coated glass coverslips in serum-free medium (DMEM:HAMS F-12) containing B27 supplement (2% v/v) but without mitogens. Over a 7–14-day period following plating, cells migrated away from the sphere and formed a differentiating neuronal and glial monolayer. The cells were fixed on day 7 or 14 in 4% paraformaldehyde and rinsed in PBS. Fixed cultures were blocked in 3% goat serum with 0.3% Triton X-100 and incubated with primary antibodies to β-tubulin III (monoclonal, 1:500, Sigma), glial fibrillary acidic protein (GFAP; polyclonal, 1:1000, DAKO), Gal-C (monoclonal, 1:300, Sigma) or tyrosine hydroxylase (TH; monoclonal; 1:500, Chemicon). Following rinsing in PBS, the cultures were incubated in either biotinylated goat anti-mouse or fluorescein-conjugated goat anti-rabbit antibodies. Biotinylated cultures were visualized using a streptavidin-rhodamine conjugate, and Hoechst 33258 was used as a nuclear stain. In order to demonstrate that differentiated cells had arisen from dividing NPCs, some neurospheres were pulsed with BrdU (0.1 μM) for 12 h prior to plating and differentiation. Following fixation, these cells were then co-stained for BrdU following the protocol supplied by a commercially available kit (Boehringer) and either β-tubulin-III, GFAP or Gal-C.

Quantification of cells migrating out from neurospheres was achieved by viewing cells under a fluorescence microscope (×40 objective) and counting Hoechst-stained nuclei along with labelled neurons in at least four independent fields (total area >0.25 mm^2) immediately adjacent to plated spheres using a pre-defined template. Measurements of neuronal cell body areas were undertaken using Openlab 2.1 digital imaging software.

2.3. [^{3}H]Thymidine incorporation assay

Single spheres were exposed to 0.5 μCi per ml of [^{3}H]thymidine for 24 h at 37°C, in the presence of growth factors as appropriate. At the end of the incubation period spheres were washed three times with DMEM and incubated for 30 min at 4°C with 10% trichloroacetic acid (TCA) to remove free [^{3}H]thymidine. The spheres were then rinsed three times with 10% TCA and washed once with 95% ethanol. The incorporated [^{3}H]thymidine was solubilized with 0.5 M NaOH for 30 min at 37°C, which was then neutralized by addition of 1 M HCl. This solution was added to 4 ml of scintillation cocktail and counted in a scintillation spectrometer.

2.4. FACS analysis

Whole neurospheres, or neurospheres plated for 7 days, were incubated in 0.1% trypsin for 20 min, washed in DMEM and then seeded into L15 medium (Gibco) supplemented with B27 (1:50) and kept at 4°C. The cells were then incubated in propidium iodide (PI) for 10 min to label dead cells and then filtered through a sterile high pass filter into a FACS analysis tube. The analysis was performed using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an argon laser emission wavelength of 488 nm as described in detail previously [25]. PI and autofluorescence was identified using a 585-nm band pass filter.

2.5. Statistical analysis

All data are expressed as means±S.E.M. and were analysed using two-way ANOVA with Bonferonni post hoc comparisons (GraphPad Prism software version 3.00).

3. Results

3.1. Regional differences in growth rates of rat neurospheres

Cells derived from either the E14 rat cortex (^{ctx}NS) or striatum (^{str}NS) grew as neurospheres following plating and showed exponential growth over the first 35 days (Fig. 1A). These numbers represent a nearly 170-fold expansion in cell number, theoretically equivalent to approximately eight population doublings. In contrast to the forebrain neurospheres, those derived from the mesencephalon (^{mes}NS) underwent an approximate three- to four-fold expansion (from 4×10^6 to $15.2\pm3.2\times10^6$) over the same period, and there was no further increase in the cell number after 21 days in culture (Fig. 1A). Post hoc comparisons between the groups revealed significant differences between effective expansion ratios for neurospheres derived from the forebrain (cortical or striatal) and midbrain ($P<0.05$) at 28 and 35 days in culture. We have previously shown that in contrast to mouse neurospheres, human and rat neurospheres could not be expanded for more than 5 weeks in culture [43,44]. We were able to overcome this growth limitation in human neurospheres by developing a chopping method of passaging which maintained cell–cell contact and allowed extended growth of human neurospheres for up to 150 days [45]. However, the same chopping method did not extend the growth of rat neurospheres. All of the rat neurospheres, regardless of regional origin, underwent senescence at 5 weeks (Fig. 1A). These results suggest that there are fundamental differences between rat and human neurospheres with regard to their continual propagation in culture using these methods.

The regional differences in rat NPC growth rates were further reflected in the relative changes in sphere size measured between day 7 and day 16 of culture (Fig. 1B). Between these time-points, the mean neurosphere diameter increased from 0.33±0.03 to 0.71±0.06 mm for ^{ctx}NS,

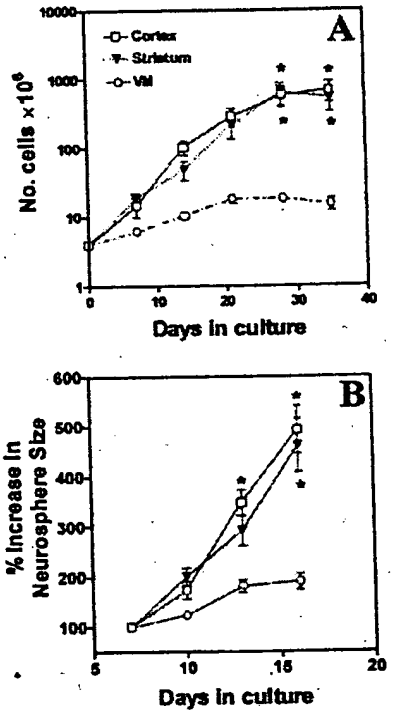

Fig. 1. Cells from the mesencephalon grow more slowly than those from the striatum or cortex. (A) Cell numbers at sequential passages. Data are means±S.E.M. for three independent experiments. Two-way ANOVA revealed a significant overall interaction between the regions across time in culture ($P<0.001$). Asterisks indicate significant difference in cell numbers between forebrain (cortical or striatal) groups and VM group at 28 and 35 days in vitro ($P<0.05$). (B) Neurosphere size expressed as percentage increase in sphere area between days 7 and 16 in culture. Data are means±S.E.M. for $n=12$ spheres per region. Asterisks indicate significant difference between forebrain (cortex or striatal)-derived neurospheres and VM neurospheres ($P<0.05$).

from 0.32±0.01 to 0.65±0.05 mm for ^{str}NS and from 0.26±0.01 to 0.35±0.03 mm for ^{mes}NS ($n=12$ spheres per region). The corresponding percentage increases in sphere size over a 10-day period also revealed significant differences between ^{ctx}NS/^{str}NS and ^{mes}NS ($P<0.05$).

3.2. Regional differences in neuronal production for rat neurospheres

Following mitogen withdrawal, whole neurospheres derived from the respective regions of the embryonic brain, and taken at sequential passages, were allowed to differentiate using an established cell migration assay [7]. In the migration assay, individual cells are required to migrate from the plated neurosphere and onto the substrate in order to be analysed. Therefore, the relative proportions of neurons, astrocytes and oligodendrocytes represent those cells that have actively migrated as progenitor cells out onto the substrate and subsequently undergone differentiation, rather than any remaining post migratory cells. After 7 days in vitro, cells that had migrated out from the neurosphere were seen to express β-tubulin-III, GFAP and Gal-C. In BrdU pulse-chase studies, many of the differentiated neural phenotypes could be co-labelled for BrdU suggesting that they had arisen de novo from the proliferating NPCs (Fig. 2). ^{Str}NS gave rise to significantly more neurons than ^{ctx}NS or ^{mes}NS at 21 days of expansion ($P<0.001$) (Fig. 3A,B). Overall, within each region, there was a trend towards a reduction in neuronal emergence with time (passage) in culture, although this result was not statistically significant. At late passages the ^{mes}NS produced very few neurons. The differences between regions for neuronal differentiation were sustained at sequential time-points in culture (Fig. 3A,B), suggesting that any regionally-defined determinants for neuronal differentiation were conserved despite on-going proliferation ex vivo. All regions produced ~10–20% oligodendrocytes at each passage, although this was not quantified in detail.

In order to determine whether migrating neurons showed distinct region specific morphologies, we assessed cell body area. Neuronal cell body areas were found to be significantly greater for neurons generated from ^{mes}NS than for neurons derived from forebrain neurospheres at 7

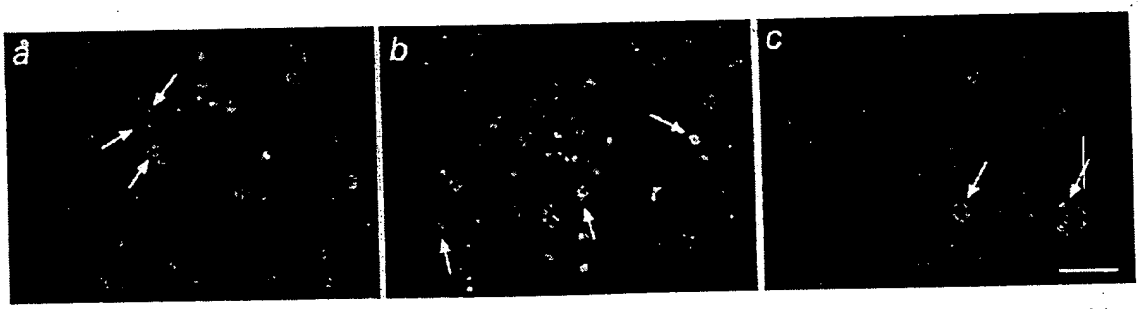

Fig. 2. Fluorescent photomicrographs showing differentiated neural phenotypes (red) emerging from whole neurospheres plated onto PLL/laminin under serum-free conditions: β-tubulin III-positive neurons (a), Gal-C-positive oligodendrocytes (b) and GFAP-positive astrocytes (c). Neurospheres were pre-pulsed with BrdU (0.1 μM for 12 h prior to plating). Cells were fixed at 7 days following plating. Newborn cells arising from proliferating neural precursor cells are double labelled for BrdU (green, arrows). Scale bar represents 50 μm.

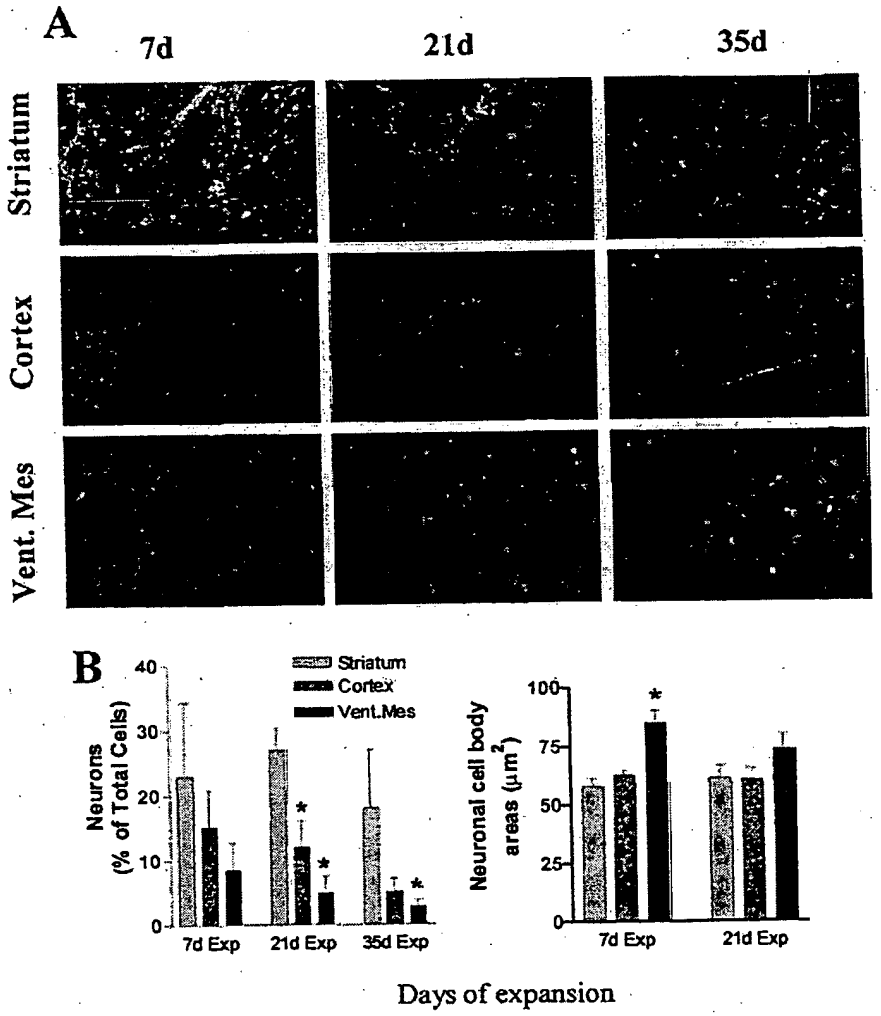

Fig. 3. Rat neurospheres are regionally specified. (A) Following differentiation at each time point, the number and phenotype of cells which had migrated out from spheres were significantly different between the striatum, cortex and mesencephalon. While the ^{str}NS generated large numbers of neurons, both the ^{ctx}NS and ^{mes}NS generated significantly fewer neurons at 21days, and the ^{mes}NS generated fewer neurons at 35 days (star, significantly different from cortex at $P<0.001$). (B) There were significantly larger cell bodies for β-tubulin III-positive neurons derived from expanded VM neural precursors when compared to neurons derived from forebrain (either cortical or striatal) precursors (asterisk indicates significance at P0, $P<0.05$). A trend for this difference was maintained at 35 days (P2). Data represent means±S.E.M. across three separate experiments.

days in vitro ($P<0.05$), and a trend for this difference was retained even after 21 days in vitro (Fig. 3B). We next assessed whether there were any specific phenotypic markers retained by the VM generated neurospheres. In particular we examined for tyrosine hydroxylase expression, which might indicate dopaminergic differentiation amongst proliferating NPCs. In keeping with our previous report [8] only a small number of well-developed TH-positive neurons were seen when neurospheres were plated following 14 days of growth. Moreover, these cells were found to reside within the limits of the neurosphere and possibly represented primary dopamine neurons which had not subsequently divided in culture (data not shown). At later passages, it was not possible to identify any TH-immunoreactive neurons in neurospheres generated from any region.

3.3. Regional specification of human neurospheres

We have previously shown that neurospheres derived from the human embryonic forebrain can be grown in culture for extended periods of time providing that cell–cell contact is maintained [45]. In the current study, human tissue from all brain regions also produced spheres after

dissociation and seeding into EGF and FGF-2 supplemented culture medium (Fig. 4A). These neurospheres continued to increase in size over time and were passaged using the chopping method. In contrast to the rat neurospheres, human neurospheres derived from all regions showed continual growth over the first 20 weeks of culture, although this was slower than that seen for the rat neurospheres. After 20 weeks of growth there were no significant differences in [^{3}H]thymidine uptake (a measure of cell proliferation) in neurospheres generated from the different brain regions (Fig. 4B). However, there was a consistent trend for the ^{mes}NS to grow at a slower rate than those from other regions. Upon mitogen removal and exposure to 1% serum and laminin, the spheres attached and cells rapidly migrated out onto the substrate. Clear differences in the total number of cells migrating were apparent in fields around neurospheres generated from each region (Fig. 4C). This was in large part due to the high proportion of small, phase bright immature neurons emerging from the ^{ctx}NS and ^{str}NS, which labelled with the neuronal marker TuJ1 (Fig. 4D,E).

Detailed analysis of the neuronal morphology revealed that cortical neurospheres gave rise to neurons that were significantly smaller than those from mesencephalic neurospheres (mean area of cortex-derived neurons±S.E.M.= 64.5±1.59, n=154 cells; mesencephalon-derived neurons=137.6±6.00, n=67 cells: significantly different at $P<0.0001$, Student's t-test). Furthermore, neurons from mesencephalic and cerebellar neurospheres often had long axonal process with characteristic blebs, not seen in neurons from cortical or striatal neurospheres (Fig. 4D, arrowheads), although very few cells from any region (<0.01%) stained for tyrosine hydroxylase. Neurons from striatal neurospheres did not stain for choline acetyltransferase or dopamine and adenosine-related phosphoprotein (DARPP-32), but many were positive for GABA and glutamate as described previously [7] suggesting only selective neurochemical phenotypes were emerging from these neurospheres. We have recently shown that addition of growth factors further increases the number of cortically derived neurons [7]. In contrast, the small number of neurons generated from hind-brain neurospheres did not

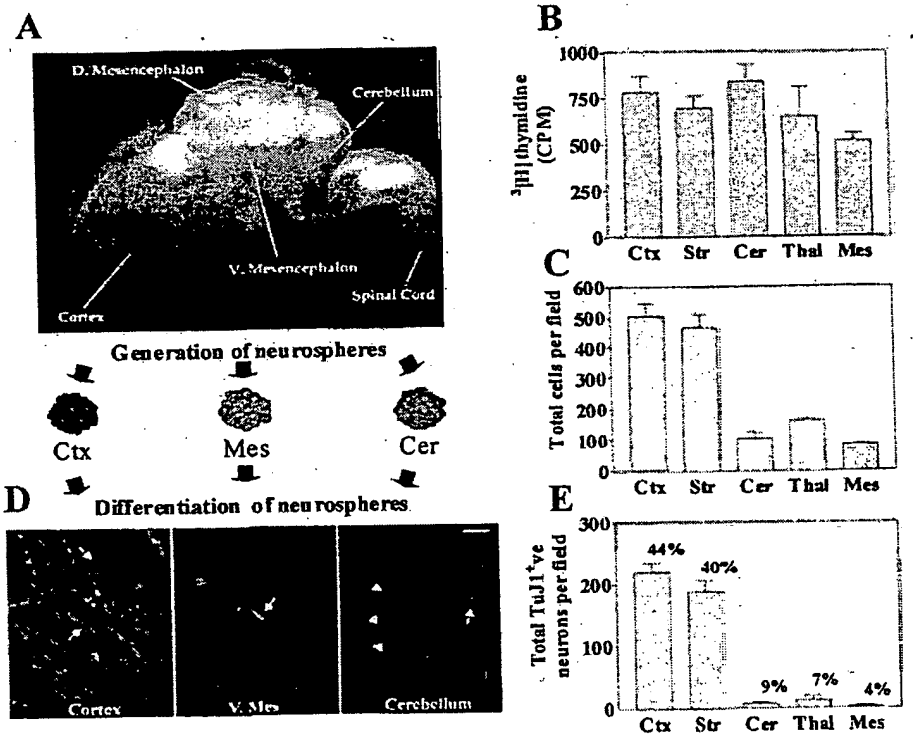

Fig. 4. Human neural precursor cells are regionally specified. (A) Brain tissue was removed from post mortem human fetal tissues (8–10 weeks post conception). (B) [^{3}H]Thymidine uptake over 24 h by neurospheres derived from the cortex (ctx), striatum (str), cerebellum (cer), thalamus (thal) or mesencephalon (mes) showed similar rates of uptake, with the lowest being the ^{mes}NS. (C) Total number of cells which migrated out from each region over 14 days. Note the marked differences in total migrating cells between the cortex and striatum compared with other regions. (D) Staining of the migrating cells from the different neurospheres with antibodies to TuJ1 (red; neuronal marker) and GFAP (green; astrocyte marker) revealed small neurons without long axonal processes from the striatum and cortex, but larger neurons with longer processes from other brain regions.. Nuclei were labelled with Hoechst (blue). (E) The cortex and striatum were unique in generating large numbers of neurons. n=3–5 separate foetal samples. Scale bar represents 50 μm.

significantly increase upon growth factor addition, suggesting that trophic factor dependent cell death is not responsible for the differences in neuronal number (data not shown). In contrast to the rodent cultures described above, only a small number of oligodendrocytes were generated from early passage human neurospheres from every region used in this study (~1%), and the number of oligodendrocytes further declined to very low levels at later passages (<0.01%).

To determine how much cell division occurs following plating, and to establish the types of cells from ^{ctx}NS that give rise to the small Tuj1 positive neurons, we used time lapse cinematography to image the cells as they emerged from the sphere. At 3 days after plating a number of cells were seen to be dividing within the region around the sphere in the absence of either EGF or FGF-2. These cells were either very small, oval migratory cells or large more static cells with a type 1 astrocyte morphology. All of the divisions within this 2D environment were symmetrical (Fig. 5; see website for live image: http://www.waisman.wisc.edu). A large cell was never seen to give rise to a small cell and large cell upon division under these conditions (over 200 divisions followed for 24 h). Thus, the large number of neurons that emerge from the ^{str}NS or ^{ctx}NS could in part be due to division of small migratory neuronal progenitors post-plating, as described previously in the rodent system [23].

3.4. FACS analysis confirms regional differences within growing spheres

We next wanted to establish the nature of the dividing cells within the human neurospheres. Since we were unable to clone single cells from these cultures (they require continual cell–cell contact to divide under the present culture conditions), and our time lapse data did not show asymmetric divisions, we were not able to prove that a single precursor was dividing asymmetrically to generate both neurons and astrocytes. It was possible that two cells were dividing alongside each other: one a small neuronal progenitor (enriched in cortical primary tissue) and the other a large astrocyte progenitor (enriched in hind-brain primary tissue). To investigate this further, a method previously developed to distinguish various cell types within rodent neurospheres based on flow cytometry (FACS) was used [25]. FACS analysis of human neurospheres based on cell size and auto fluorescence revealed that there was a population of small, weakly fluorescent cells found only in neurospheres derived from the forebrain (Fig. 6A, R3). Following differentiation for 7 days, the proportion of small cells increased dramatically in forebrain neurospheres (Fig. 6B, R3). In addition to the increase in this small cell population, the cells themselves were apparently smaller. Similar populations of small cells were absent from the ^{mes}NS (Fig. 6B, R3). These results

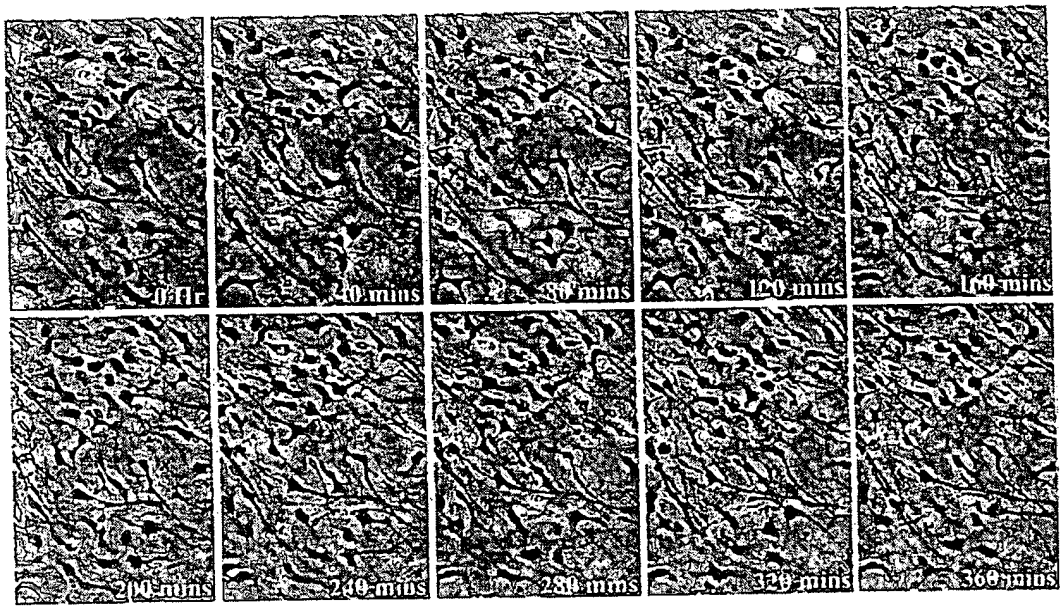

Fig. 5. Time lapse recording of cells around a human neurosphere dividing and migrating. Spheres were plated onto laminin in the absence of EGF and allowed to differentiate for 3 days. Interestingly, even in the absence of mitogens some division continued post plating. (A) Representation of a small migratory neuroblast which would invariably be TuJ1 positive (Fig. 7). (B) Representation of a small migrating neuroblast which underwent cell division (arrows) to give rise to two anatomically similar daughters which then migrated away. (C1 and C2) Representation of two large cells with an astrocytic morphology which underwent cell division (arrows) and gave rise to very similar large daughter cells. These large cells were far less migratory than the smaller ones. A video sequence can be seen in the on-line version of the paper, available on http://www.neuroscion.com.

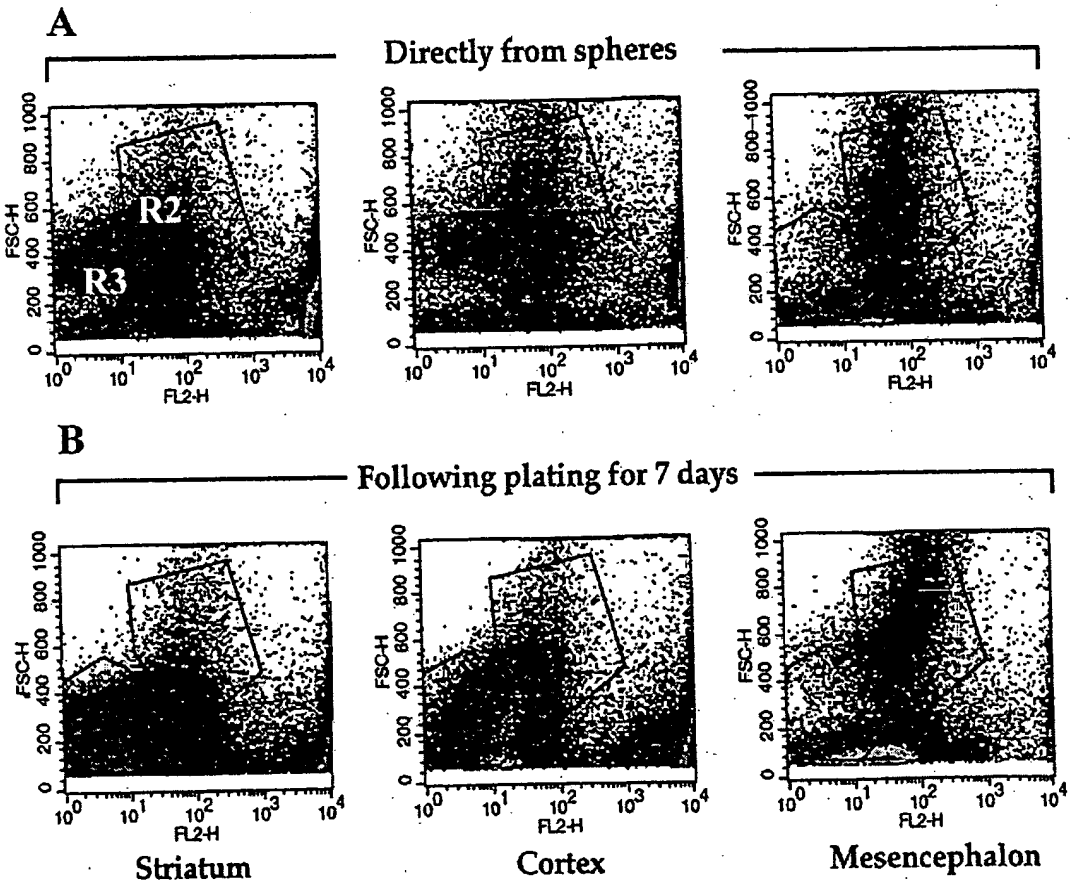

Fig. 6. FACS analysis confirms regional differences are apparent even within neurospheres. (A) Human neurospheres from different brain regions grown for 20 weeks as described in the Materials and methods section were dissociated and stained with propidium iodide, which labels dead cells, and sorted by size (*Y*-axis) and intensity of fluorescence (*X*-axis). Dead cells appear as highly fluorescent band to right of plot. Each dot represents a single cell. Neurospheres from the cortex and striatum showed a side band of smaller, less fluorescent cells (in region R3) which were missing in neurospheres from the mesencephalon. (B) Following differentiation for 7 days, the ^{ctx}NS and ^{str}NS gave rise to many small cells which appear in R3. These were not seen to develop from the mesencephalon derived neurospheres. This analysis was repeated using three separate cultures with similar results.

therefore suggest that these cells represent the large number of small neuronal progenitors seen to arise from these cultures, and shown to be actively migrating in Fig. 5.

To determine whether the unique population of small cells in neurospheres derived from forebrain were indeed small self-renewing neuronal progenitors, cells from human ^{ctx}NS were sorted and separated in to large (R2) and small (R3) populations (Fig. 7A). These cells were either plated onto laminin to assess differentiation, or seeded at high density with EGF to generate new neurospheres. After plating onto laminin, R2 cells showed a large amorphous phenotype, and a number of these had already begun to express GFAP after only 24 h (Fig. 7B). By 7 days nearly all of the cells in these cultures were GFAP positive (not shown). No Tuj1^{+}ve neurons were detected in these cultures at any time point. In contrast, after plating onto laminin the small R3 cells were enriched for TuJ1^{+}ve neurons while still containing a few GFAP^{+}ve astrocytes (Fig. 7B).

Did the large cells, which form mostly astrocytes and no neurons following plating onto laminin (presumably due to not undergoing asymmetric divisions), have the potential to generate neurons? To test this we attempted to make new neurospheres from the sorted cell populations. Following re-exposure to EGF immediately following plating onto non-coated plastic dishes, the small R3 cells failed to generate any new neurospheres (Fig. 7C). In contrast, the large R2 cells plated at high density in the presence of EGF rapidly aggregated and formed new spheres (Fig. 7C). Following 14 days of growth, intact spheres derived from the large R2 group were re-plated as whole spheres onto laminin and allowed to differentiate. Within 7 days they had generated large numbers of small TuJ1^{+}ve

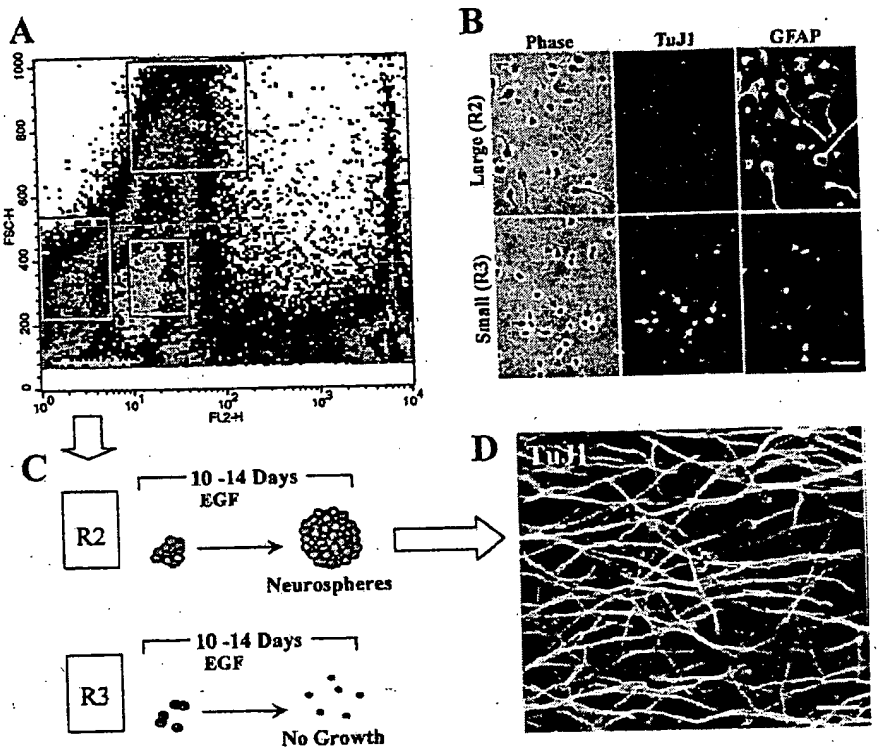

Fig. 7. A large cell with a glial morphology can give rise to neurons. (A) Neurospheres generated from the human cortex and grown for 20 weeks were sorted by FACS and two regions collected (large R2 and small R3). (B) Following acute plating (2 h) onto coated wells, the small R3 cells gave rise to a high numbers of Tu1 positive neuroblasts (arrows) with a few GFAP positive cells (arrowheads). In contrast the large R2 group gave rise to both GFAP (arrowheads) and unlabelled cells but no Tuj1 positive neuroblasts. Scale bar represents 80 μm. (C) Following re-exposure to EGF at high plating densities the R2 group generated new neurospheres whereas the R3 group did not respond to EGF. (D) Following 2 weeks of expansion, neurospheres generated from the R3 group again generated large numbers of small Tuj1 positive neurons following plating for 7 days. Scale bar represents 50 μm. This analysis was repeated twice with two separate cultures with similar results.

neurons (Fig. 7D). This demonstrates that the large cell population within the ^{ctx}NS is the neurosphere forming cell. Furthermore, this cell is able to generate a new population of small neuronal progenitors within the 3D environment of the neurospheres.

3.5. Discussion

3.5.1. Limited growth of rat but not human neurospheres

In this study, and in our previous report [44], we were unable to grow rat ^{str}NS for more than 6 weeks. Various techniques shown to enhance the growth of rat neurospheres, such as maintaining cell–cell contact, adding heparin to the medium, combining EGF and FGF or adding LIF to the medium (reviewed in Ref. [41]) did not overcome this lack of growth in the current study. The growth restriction was not simply a function of embryonic age, as a similar cessation in growth occurs in neurospheres derived from tissue between E12 and P1 (Rossor and Svendsen, unpublished observations). Neurospheres generated from other brain regions also stopped growing after a short period of expansion. Paradoxically, rodent neurospheres express high levels of telomerase and have long telomeres [29], suggesting that they have enormous potential for growth. Human neurospheres, on the other hand, express only small amounts of telomerase and have shorter telomeres, yet grow for much longer periods [29]. Clearly in neurospheres derived from different species, there are cell proliferation checkpoint mechanisms that are independent of telomerase activity. Furthermore, it is likely that the culture conditions for rat neurosphere growth have not yet been optimised, and that their early cessation of proliferation could be overcome in the future. Indeed, rodent oligodendrocyte precursor cells, which normally can only undergo eight divisions in culture, can continue to divide for extended periods of time providing thyroid hormone is removed from the media [46]. We look forward to future improvements in the growth of rat neurospheres as well.

3.5.2. Regional specification of neurospheres in the absence of environmental signals

Classical retroviral studies have shown that at early stages of development, at least some single labelled cells can give rise to multiple types of neuron and glia [37]. Thus, there is widespread agreement that true multipotent neural stem cells exist in vivo. However, similar studies have also shown that within the developing cortex, many cells are more restricted progenitors, such as those giving rise to either pyramidal or non-pyramidal neurons [30]. Furthermore, cortical and striatal progenitors retain the capacity to differentiate into specific phenotypes, even when removed from their in vivo environment and induced to divide several times in vitro [14,38]. The fate of cortical neurons appears to be critically dependent on the signals the cells receive in the final stages of cell division [24]. In the neurosphere model used in the present study, the environmental cues are limited to the surrounding cells, all undergoing synchronous differentiation. These surroundings may be very different to the in vivo situation where polarity, growth factor gradients and a temporally defined range of different cells are influencing cell fate. Under the culture conditions employed here, all neurospheres gave rise to differentiating neurons, although the number and morphology were very different. Since at least some of these neurons had arisen from dividing neural precursors, as indicated by BrdU co-labelling, the regional differences in neuronal emergence are most unlikely to reflect differences amongst post-migratory neurons remaining from the primary culture. Rather, they are likely to reflect the presence of regionally-specified cell-autonomous signals retained within neurospheres.

The migrating neuroblasts from the ^{mes}NS were consistently larger than those from the ^{str}NS or ^{ctx}NS and often had long axonal projection fibres. We propose that in the absence of other signals, there must be a program within the dividing and migrating progenitor cells which determines cell size and differentiation. As such they might be considered lineage restricted, perhaps in a similar fashion to the long-term and transiently self renewing populations of hematopoietic stem cells described previously [26]. Alternatively, the surrounding migrating glial cells may release factors that affect the size and number of migrating neuroblasts. It of course remains possible that under different culture conditions, or following grafting, the fate of these progenitor cells may be changed, i.e. they are not determined, but specified in the absence of other signals. In addition, there may be a few true multipotent, or even pluripotent stem cells in neurospheres from each brain region dividing slowly alongside more rapidly proliferating restricted neural progenitors (Fig. 8). These would be missed when large numbers of cells are plated simul-

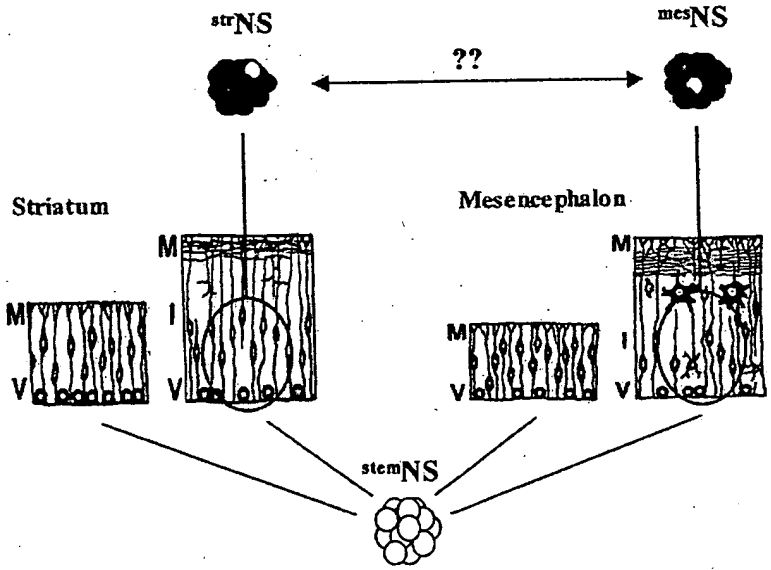

Fig. 8. Neurospheres are regionally specified. Our initial prediction was that perhaps a common cell could be isolated from all regions of the developing brain and grown into a neurosphere (^{stem}NS in white). Instead, the majority of cells within the spheres had regional specificity, even after extended passaging. Those from the striatum (red) had a greater capacity to migrate out from the sphere and form neurons when compared to those from the mesencephalon (green). Our data do not exclude the possibility of non-specified, equipotent stem cells existing within spheres from both regions in very small numbers (white), which exist in ventricular zones (V) throughout the developing neuroaxis. This cell may be masked by the many specified cells. Nor do we know whether under different environmental conditions the fate of spheres from each region might be altered (dotted line). However, we hypothesize that the majority of cells within neurospheres arise from regionally specified progenitors within the intermediate zone (I). Further studies are now required to establish the mechanisms underlying these observations.

taneously. It is possible that these pluripotent stem cells may be responsible for the reported trans-differentiation of human neurosphere derived cells into muscle cells at low efficiency [18].

It is tempting to speculate that the relatively high proportion of neurons arising from rat ^{str}NS reflects the protracted period of neurogenesis in the rat striatum, extending from embryonic day 12 (E12) to E22 with a peak at E15/E16 [15]. In comparison, cortical neurogenesis in the rat occurs during a more restricted 6-day period from E13.5 to E19.5 [4]. Similarly, the neurons of the rat substantia nigra are generated from dividing precursor cells in the ventral mesencephalon over only 4–5 days, between E11 and E15 [1,19,21]. These features of neural development are consistent with the present findings that the ^{str}NS gave rise to more neurons than those from either ^{mes}NS or ^{ctx}NS. Thus, the rat neurosphere culture model may more closely resemble in vivo neural development than had previously been realized. The human neurosphere cultures were also regionally specified, but had a far greater capacity for division. Human neural development takes place over at least 9 months with complex patterns of division and migration for each brain structure. It is possible that the protracted division compared to the rat also represents a greater capacity for human cells to divide in vitro and produce the greater quantity of neurons found in the human brain compared to the rodent. We are currently further studying mechanisms of continual proliferation in these human neurosphere cultures.

It was notable that, despite the novel passaging techniques used here for optimising cell–cell contact between proliferating NPCs, the ^{mes}NS only gave rise to very small numbers of TH-positive neurons in comparison to primary mesencephalon cultures. This is consistent with the data suggesting that embryonic dopamine neuroblasts have a limited capacity to undergo in vitro proliferation and expansion [6,8,39]. Indeed, it is recognised that the effective in vitro expansion and/or induction of the DA phenotype in propagated NPCs is likely to require their genetic modification in combination with the appropriate extrinsic inductive signals [50]. Therefore, while there are clear regional differences in the numbers and size of neurons, the generation of neurochemical phenotypes specific to each region is likely to require additional signals which are not present in our culture system. It is of interest that the human cerebellar and thalamic derived neurospheres also generated low numbers of neurons. Based purely on developmental maturation, the cerebellum might be expected to contain the most primitive type of stem cell, and to retain the capacity to produce high numbers of neurons due to the enormous volume of cerebellar granular cells found in the mature human brain. However, this was not the case. It might be that there is an optimal developmental window for harvesting human neural cells capable of making neurospheres with a high neuronal differentiation potential, and that this may differ between brain regions. We are currently investigating whether this is in fact the case.

3.5.3. The special capacity of forebrain neurospheres to generate high numbers of neurons

Why should the neurospheres derived from the human forebrain produce such high numbers of small neurons even after extensive passaging, when the mesencephalon, thalamus and cerebellum produced relatively few? The sub-ventricular zone (SVZ) of the adult rodent forebrain has been shown to harbour cells expressing astrocyte markers (type B cells) which are capable of producing neuronal progenitors (type A cells) via an intermediate cell (type C cell [13]). The type A neuronal progenitors then migrate along the rostral migratory pathway into the olfactory bulb [22]. This three cell system, where the type C cell is capable of generating large numbers of small migrating neurons, is not only unique to the mammalian forebrain, but is also absent in hindbrain structures which do not have an SVZ or in avian species. A very similar three cell system has recently been described in the primate forebrain [20]. We have shown through FACS analysis that a large cell within the forebrain neurospheres, which upon acute differentiation and plating exclusively generated astrocytes, was able to generate neurons when re-exposed to EGF and expanded again in neurosphere cultures. It is possible that this cell may be a type B cell, which within the three dimensional environment of the neurosphere is able to generate type C cells that in turn produce small type A neuronal progenitors. This cell may not be present in neurospheres derived from other brain regions, which were only able to generate neurons directly from the type B cells with no type C intermediate. Although this hypothesis remains highly speculative, it is worthy of further investigation and provides at least one potential mechanism by which the cortical and striatal human cultures are able to maintain such a high neuronal output.

We show here that neurospheres generated from all regions of the human brain were able to produce both astrocytes and neurons, but very few oligodendrocytes at later passages. The lack of oligodendrocyte production seen here and by others at late passages using similar culture systems [10,48] suggests perhaps that either (i) the methods used to grow long-term neurosphere cultures favour committed neuron/astrocyte progenitors rather than multipotent neural stem cells or (ii) they are all multipotent stem cells, but the culture conditions are not correct to produce oligodendrocytes. It is very difficult to distinguish between these two possibilities until we understand more about these cells. However, there is again a clear species difference in that the rodent cultures described produce many oligodendrocytes even if generated from regions of the spinal cord not expected to produce them [11]. A lack of oligodendrocytes does not imply that they could not be

generated from these human cultures under the correct culture conditions, or following transplantation. However, we have so far failed to achieve this effect using a range of different paradigms (Chandran and Svendsen, unpublished observations).

3.5.4. Implications for cell therapy using human neural stem cells

The data presented here suggest that there are significant regional differences when neurospheres are derived from different areas of the developing rodent or human brain. Thus, many of the cells dividing within neurospheres retain a memory of their origin. This may be a result of the different developmental stages the regions were at when cultured, or simply that each neurogenic zone of the developing brain has a specific type of cell which responds to EGF and FGF-2. Regardless of the mechanism underlying this phenomenon, it will be important for transplantation studies using cells derived from human neurospheres to establish their exact origin. It is possible that those from regions other than the striatum or cortex are not likely to generate large numbers of neurons following transplantation, although the effects of different environmental signals now need to be investigated. Hindbrain neurospheres produce less neurons, but those that were generated were larger and had longer processes. Thus we speculate that while cortical/striatal neurospheres may be useful for replacing lost inter-neurons, hindbrain neurospheres may be better suited to replacing large projection neurons. However, this remains to be established in transplantation models.

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EXHIBIT F

The development of neural stem cells

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The discovery of stem cells that can generate neural tissue has raised new possibilities for repairing the nervous system. A rush of papers proclaiming adult stem cell plasticity has fostered the notion that there is essentially one stem cell type that, with the right impetus, can create whatever progeny our heart, liver or other vital organ desires. But studies aimed at understanding the role of stem cells during development have led to a different view — that stem cells are restricted regionally and temporally, and thus not all stem cells are equivalent. Can these views be reconciled?

The discovery of neural stem cells was rooted in classic studies of haematopoiesis and of invertebrate neural development, which inspired examination of single neural progenitor cells. (In this review, I will use the term 'progenitor cell' to refer to all classes of immature, proliferating cells. Neural stem cells are a subtype of progenitor cells in the nervous system that can self-renew and generate both neurons and glia.) The early studies led to the isolation of stem-like cells from the embryonic mammalian central nervous system (CNS)[1–4] and the peripheral nervous system (PNS)[5]. Since then, stem cells have been isolated from many regions of the embryonic nervous system, indicating their ubiquity (Fig. 1a). After the discovery of neural stem cells in the embryo, the first isolation of stem-like cells from adult brain[6,7] began yet another chapter of neuroscience. Adult neural stem cells have now been found in the two principal adult neurogenic regions, the hippocampus and the subventricular zone (SVZ), and in some non-neurogenic regions, including spinal cord[8–10] (Fig. 1a). These pioneering studies provided a cellular mechanism for adult neurogenesis, which was well-established in birds and becoming accepted in mammals, and raised the possibility that the most intractable of tissues — the CNS — might have regenerative powers.

Markers that define CNS stem cells are only now being developed[11–14]. Hence, they are usually identified retrospectively on the basis of their behaviour after isolation. In adherent cultures, CNS stem cells produce large clones containing neurons, glia and more stem cells; they can also be cultured as floating, multicellular neurospheres[8–10]. PNS neural crest stem cells express the low-affinity neurotrophin receptor p75 (ref. 5), and grow as adherent clones containing peripheral neurons and glia, smooth muscle cells and more stem cells[15].

This review summarizes what we currently know about stem cells in the developing nervous system, and evaluates the idea that embryonic neural stem cells are heterogeneous and restricted. Studies that indicate broad plasticity of adult

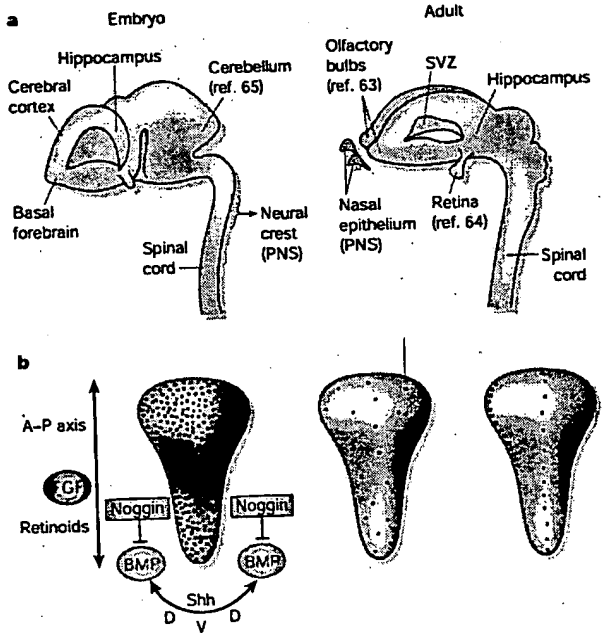

Figure 1 The location of neural stem cells. a, The principal regions of the embryonic and adult nervous system from which neural stem cells have been isolated[9,10,63–65]. b, Three models describing stem cells in the vertebrate neural plate. All neural plate cells are stem cells (left), or stem cells are a minor population that is evenly distributed (middle) or located in particular regions such as the midline and lateral edges (right). Factors such as bone morphogenetic proteins (BMPs), Noggin, retinoids, Sonic hedgehog and fibroblast growth factors (FGFs), which provide anterior–posterior (A–P) and dorsal–ventral (D–V) patterning information, may regionalize stem cells[24].

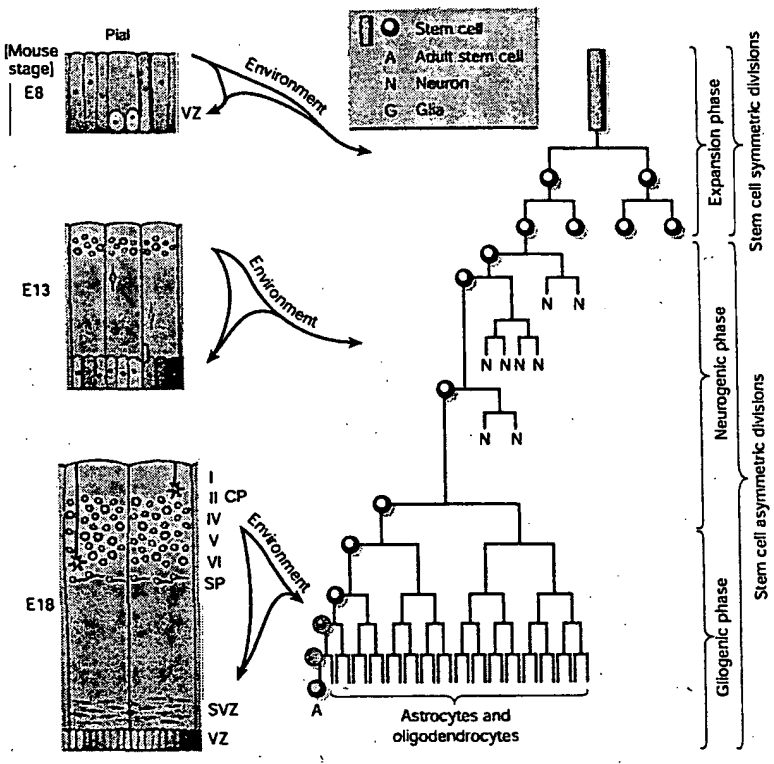

Figure 2 The development of stem cells in the mammalian CNS. Alignment of *in vivo* developmental events with *in vitro* behaviour of stem cells derived from embryonic forebrain has led to the following concept of how neural stem cells change over time. Early neuroepithelial cells are columnar, touching ventricle and pial surfaces during the cell cycle. At mid-gestation, young neurons have migrated above the germinal ventricular zone (VZ), radial glia continue to contact both ventricle and pia, guiding neuronal migration, and a second germinal zone arises, the subventricular zone (SVZ). By postnatal ages, radial glia have transformed into astrocytes and the VZ also disappears, but the SVZ remains into adulthood in some areas. Stem cells present in the early cerebral cortical neuroepithelium divide symmetrically first, and then asymmetrically to generate differentiated progeny. Neurons are produced first, and migrate along radial glia up towards the pial surface where they settle in the subplate (SP) and the cortical plate (CP). After the neurogenic period, the stem cell makes glial progenitor cells that proliferate largely in the SVZ. By birth, the stem cell has developed and has different characteristics, such as responses to growth factors, to those of the original embryonic stem cell. Stem cell development might be driven by a combination of intrinsic temporal programmes and extracellular signals from the changing environment of the developing brain.

stem cells are also discussed. By examining these two different aspects of stem cell research, future directions of exploration are highlighted that might help explain this apparent dichotomy.

Stem cells at the beginning of the nervous system

Many fundamental questions regarding specification of early neural stem cells remain unanswered. When the neural plate first emerges, does it consist solely of stem cells or does it include both stem cells and restricted progenitor types (Fig. 1)? Analysis of adherent clone production suggests stem cells are prevalent at early stages. In spinal neural tube from embryonic day 8 (E8) rat, over 50% of the viable cells at 24 hours are stem cells[16,17]. In telencephalon from E10 mouse, estimates of stem cells range from 5 to 20% (refs 4, 18, 19). Most of the premigratory neural crest consists of stem cells[20].

But the frequency of stem cells declines rapidly, diluted by the production of restricted progenitors and differentiated cells; for example, in spinal cord it drops to 10% at E12 and 1% at postnatal day 1 (P1)[16,17]. Notably, stem cells seem to be much rarer when neurosphere production is used as the assay: only 0.3% of E8.5 mouse anterior neural plate cells make neurospheres[21]. Perhaps neurosphere-generating cells are a subpopulation of early stem cells, or perhaps stem cells are more prevalent in spinal cord than anterior regions at this age.

How are neural stem cells initially specified? The stem cell could be the default state or, alternatively, stem cells might be induced. Pluripotent embryonic stem cells can produce a primitive type of neural stem cell when grown in isolation, but only 0.2% of embryonic stem cells generate neurospheres[22]. Although this study suggests that there is a default pathway for acquiring the stem cell state, the low frequency indicates that it may be normally enhanced by inductive mechanisms.

If stem cells are, or rapidly become, a subset of early neural progenitor cells *in vivo*, how are they distributed (Fig. 1)? Without specific markers, important questions regarding stem cell frequency and location *in vivo* remain open.

The early, widespread presence of stem cells in the embryonic nervous system raises another important issue of their role in development. Given their prolific, multipotent nature *in vitro*, they are likely to be principal progenitors *in vivo*, but this remains to be shown directly. For example, it is possible that early restricted neuroblasts rather than stem cells might generate the preponderance of neurons. Clonal studies suggest that most glia, both astrocytes and oligodendrocytes, originate from stem cells[9,18,19], signifying their importance for gliogenesis. In fact, as described below, there may be an intimate association between glia and the neural stem cell state.

Neural stem cells acquire positional information

Patterning of the body axis occurs through signalling systems that impart positional information. For example, gradients of signalling molecules can regionally specify a population of progenitor cells if the cells respond differently to different concentrations of the signal[23]. In the nervous system, the salient patterning in anterior–posterior and dorsal–ventral axes occurs early, concomitantly with neural induction (Fig. 1)[24].

Do both stem cells and restricted progenitor cells exhibit regionalization, or do stem cells remain unspecified, maintaining their plasticity? In *Drosophila* and grasshopper, each stem cell-like neuroblast has a unique identity based on its position in the neuroectoderm[25]. In vertebrates, this question is just beginning to be explored. Neurospheres generated from different CNS regions express region-appropriate markers, indicating that the original stem cells were indeed regionally specified[26]. Regulatory sequences control region-specific expression of the transcription factor Sox2, so that expression is seen in telencephalic but not spinal cord stem cells[27].

Table 1 Summary of transplantation studies assessing stem and progenitor cell behaviour

Donor	Host site	CNS incorporation	Differentiation	References
Embryonic stem cells				
Mouse ES cells or ES cell-derived neurospheres	Blastocyst	Incorporation into all embryonic tissues	Both glutamate- and GABA-mediated neurons in cortex	50,51
Mouse ES cell-derived neural precursors	E16–18 rat LV	Widespread incorporation	Neurons, oligodendrocytes and astrocytes	52
Embryonic neural cells				
E9.5 and E14.5 forebrain-derived neurospheres	Blastocyst or morula	No aggregation		22
EGF-generated neurosphere cells from E14 mouse fore- and midbrain	E15 rat LV	Fore- and midbrain structures	Astrocytes	53
Fetal human brain-derived neurospheres or primary cells	E17–18 rat or P0 mouse LV	Widespread incorporation in the brain	Neurons (projection), oligodendrocytes and astrocytes	54,55
E10.5 mid/hindbrain	E13.5 MGE	Dispersed into forebrain	Site-specific neuronal differentiation	31
E12–14 mouse forebrain	E15 to P1 rat LV	Into forebrain and midbrain; incorporation and migration reduced as host age increases	Site-specific neuronal differentiation	56,57
E13.5 mid/hindbrain	E13.5 LGE or MGE	No integration into forebrain		31
E36 ferret cortex (making layer 4)	E30 ferret (making layer 6)	Neocortex and hippocampus	Layers 2–5; no transplanted cells in layer 6	29
	P2 (making 2/3)	Neocortex and hippocampus	Layer 2/3 neurons	
Early postnatal				
Postnatal mouse SVZ cells	E15 mouse LV	Septum, thalamus, hypothalamus and inferior colliculus; not cortex or hippocampus	No principal projection neurons	32
Neonatal mouse SVZ neurospheres	Chick embryo	Migrate in isolation	Neural crest derivatives	46
Neonatal mouse SVZ cells	Chick embryo	Chain migration in the neural crest pathway	Progenitors and neurons; not neural crest phenotypes	46
	Neonatal striatum	Migration	Neuronal precursors and olfactory bulb neurons	58
P0–5 mouse SVZ cells	Adult striatum	Some migration	Olfactory bulb interneurons	59
Adult				
Mouse forebrain neurospheres	Blastocyst or morula	No aggregation		22
	Mouse morula or chick embryo	Rare neural chimeras	Cell types not reported	60
Mouse SVZ neurospheres	Chick embryo	Migrate as isolated cells	Phenotypes of neural crest derivatives	46
Mouse SVZ cells	Chick embryo	Cells die		46
	Adult mouse striatum	Minimum migration	Some neurons, mostly astrocytes in striatum	61
	Adult olfactory bulb	Extensive migration	Olfactory bulb neurons	61
Cultured rat hippocampal progenitors	Adult rat hippocampus	Migrate	Neurons in granule cell layer of dentate gyrus	42, 62
	Adult rat rostral migratory stream	Migrate to olfactory bulb	Neuroblasts and olfactory neurons	42
	Adult rat cerebellum	Incorporation	No neurons	42
FGF-responsive rat spinal cord progenitors	Adult hippocampus	Broad dispersion	Hippocampal granule neurons	41
	Adult spinal cord	Broad dispersion	No neurons	41

These studies indicate stage-dependent restrictions in the potential of donor progenitor populations (which include some neural stem cells). They also emphasize the impact of interaction between implanted cells and the host environment. Different treatments of stem and progenitor cells *in vitro* can significantly alter their behaviour after implantation. EGF, epidermal growth factor; ES, embryonic stem; FGF, fibroblast growth factor. LGE/MGE, lateral/medial ganglionic eminence; LV, lateral ventricle.

In addition, stem cells isolated from different neural regions generate region-appropriate progeny. Spinal cord stem cells generate spinal cord progeny[17]. Basal forebrain stem cells cultured at clonal density generate significantly more GABA (γ-amino butyric acid)-containing neurons, which are characteristic of basal regions, than dorsal stem cells cultured under identical conditions[19]. PNS neural crest stem cells, which arise at the lateral edges of the neural plate, express distinct genes and generate progeny distinct from those of CNS stem cells[15,28]. Hence, vertebrate stem cells seem to be positionally specified.

Neural stem cells acquire temporal information

Different neural cell types arise in a precise temporal order that is characteristic for a particular region and species. In general, CNS and PNS neurons arise before glia, and specific types of each cell have specific birthdates. Timing seems to be encoded in progenitor cells, so that besides positional information they have 'temporal information', which is seen as stage-dependent changes in progenitor cells (Table 1). Thus, late-embryonic ferret cortical progenitor cells cannot make cells appropriate for younger stages when transplanted into early cerebral cortex[29]. Rat cortical progenitors become restricted in their ability to generate limbic system-associated membrane protein (LAMP)-positive limbic cortical progeny after E14 (ref. 30). Mid/hindbrain progenitor cells are unable to generate telencephalic phenotypes after E13.5 in mouse[31]. SVZ cells can no longer make projection neurons by birth[32,33], and retinal progenitor cells seem to be similarly restricted temporally[34,35].

Stem cells are a minor component of the progenitor population in these studies, but experiments indicate that they also exhibit stage-dependent changes in potential. Some early neural tube cells produce both CNS and PNS stem cells, suggesting that there is a common progenitor for two separate stem cell lineages with more restricted potentials[28]. Each of these lineages also changes over time. CNS stem cells undergo repeated asymmetric cell divisions, first producing neurons then glia[18], thus reproducing the normal neuron–glia order (Fig. 2). Moreover, they have an active role in this process by altering their intrinsic properties. Thus, stem cells from earlier stage cortices

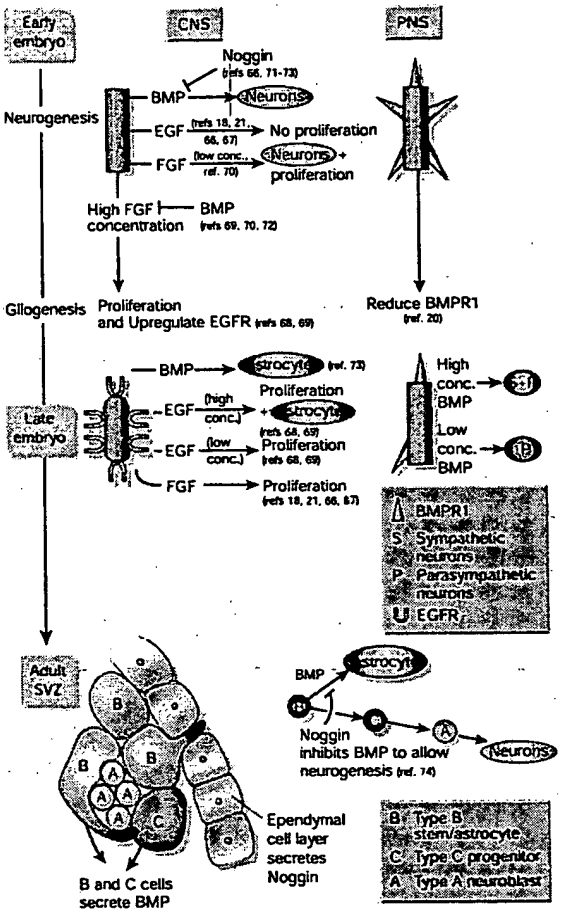

Figure 3 Stem cells alter their responses to growth factors over time. Central and peripheral nervous system (neural crest) stem cells extracted from different ages show differing responses to application of the same growth factor. Changes in levels of growth factor receptors have been observed. In addition, stem cells exhibit different responses to growth factors applied at different concentrations. BMP, bone morphogenetic protein; EGF, epidermal growth factor; EGFR, EGF receptor; FGF, fibroblast growth factor.

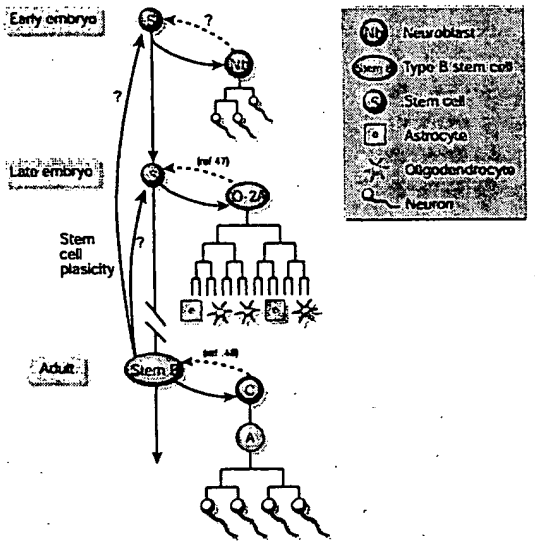

Figure 4 Can the stem cell lineage be reversed? Evidence indicates that neural stem cell progeny can reacquire stem cell properties. An important issue is whether adult stem cells can revert to an earlier embryonic state, with a wider potential.

produce more neurons and have a lower tendency to produce glia than those from later stages.

Temporal specification of stem cell populations also occurs in the PNS. Mouse neural crest stem cells isolated from the rat neural tube have been compared with later stem cells isolated prospectively from E14 sciatic nerve after transplantation into different sites of the chick embryo[20]. Early neural crest stem cells generate significantly more neurons than later stage cells: like CNS stem cells, their neurogenic capacity declines with stage. Furthermore, the range of neurons generated by late neural crest stem cells is more restricted. Early transplants that are highly enriched for neural crest stem cells but contain some restricted sensory progenitor cells can generate dorsal root ganglion (DRG) sensory neurons, and adrenergic and cholinergic autonomic neurons. By contrast, older-stage neural crest stem cells made no DRG neurons, only rare adrenergic sympathetic neurons, but they could generate cholinergic autonomic neurons.

Stem cells also undergo phenotypic changes as germinal zones develop (Fig. 2). It has been suggested recently that some radial glia present at mid-gestation might be stem cells (reviewed in ref. 36). This idea is appealing, given that radial glia are thought to be neurogenic precursors in adult canary brains, and that astrocytes, the lineal descendants of radial glia, have stem cell properties in the adult mammalian SVZ[37].

Developmental changes in stem cells — for example, in their potential and phenotype — are accomplished by, and perhaps driven by, changes in their growth factor responsiveness (Fig. 3; and see accompanying reviews by Spradling and colleagues, pages 98–104, and Weissman and colleagues, pages 105–111). Thus signalling molecules, such as fibroblast growth factors, bone morphogenetic proteins and Noggin, can influence neural stem cells from neural induction through adulthood, but their responses to these factors vary with stage.

The mechanisms underlying temporal changes in neural stem cells are not understood. The lineage trees of mouse CNS stem cells are remarkably similar to those of invertebrates[38]. *Drosophila* CNS neuroblasts express sequentially the transcription factors Hunchback, Krüppel, POU-domain genes 1 and 2, Castor and grainyhead, which specify the production of different neurons at different times[39,40]. The expression sequence may be driven by a cell-intrinsic clock[40]. Perhaps similar intrinsic timing mechanisms, combined with environmental input, temporally specify mammalian CNS stem cells. Growth factor concentrations vary during development and neural stem cells respond differently to different concentrations of the same molecule (Fig. 3). Hence, it is tempting to speculate that, just as positional information can be imparted by spatial gradients of signals[23], temporal information might be imparted by temporal gradients of signalling molecules.

Examining the plasticity of adult stem cells

If stem cells undergo developmental changes, adult stem cells are likely to differ from those in the embryo and to be regionally and temporally restricted.

One way to examine the plasticity of adult neural stem cells is to transplant them directly into the developing embryo and examine

Box 1
Potential therapeutic uses of stem cells to repair the nervous system

Stem cells are under active consideration as a source of donor tissues for neuronal cell therapy[75].

Parkinson's disease. The requirement is to generate cells that synthesize and release dopamine for implantation into the dopamine-depleted striatum. For this therapy to be effective, it is unknown whether these cells must also mature into projection neurons with synaptic host connections — a process that is required for optimal effects of embryonic nigral grafts.

Huntington's disease. If we can control differentiation into mature neuronal phenotypes then many other diseases that involve loss of specific neuronal types, such as the striatal medium spiny projection neurons lost in Huntington's disease, might be suitable for transplantation of neurons expanded from stem cell sources.

Spinal cord injury. Stem cells may be able to repopulate the site of injury, provide a substrate for axon growth across the transection, and block syrinx progression. Each of these effects has been found with primary embryonic cells; however, studies using stem or immortalized precursors are still preliminary.

Stroke. Stem cells and immortalized precursors may be able to migrate through the central nervous system and repopulate sites of ischaemia. In spite of rather limited evidence from animal studies, clinical trials of this strategy are already underway.

Multiple sclerosis. Oligodendrocyte lineages are better characterized than neuron lineages, and oligodendrocyte precursors can differentiate and provide a functional remyelination of axons after focal experimental demyelination. For application in multiple sclerosis, the main problem is how to stimulate the migration of such cells to diverse sites of demyelination that occur sporadically in the human disease. Notwithstanding the potential applications of oligodendrocyte lineages in several diseases, many key technical problems remain to be resolved.

Sources. If we select early embryonic stem cells, then the number of transformations and the complexity of signals required to achieve a specific differentiated phenotype may prove prohibitive; instead, it may be easier to control the phenotypic differentiation of developing neuronal precursors, but that might in turn limit their capacity for expansion. Adult-derived cells may circumvent both ethical and immunological constraints, but their plasticity for expansion and differentiation remains to be established. Cross-lineage transformation offers a new prospect for a more flexible source, in particular to derive autografts from patients themselves. A further advance is that precursor cell are less immunogenic than primary embryonic neurons in xenografts, highlighting a way to overcome one of the main difficulties of transplantation from non-human donors.

Expansion. Neuronal stem cells from species other than mice seem to senesce on repeated passage, with only limited potential for expansion. Conversely, if not fully differentiated at the time of implantation, there is always the possibility of tumour formation — a problem that is still not resolved for either embryonic stem cells or immortalized precursors.

Differentiation. The biggest single problem still to be solved is how to direct and control the differentiation of specific target phenotypes required for replacement and repair in each disease. Selection of appropriate starting cells by embryonic regional dissection and stage of development, as well as diverse parameters of *in vitro* manipulation, are likely to be crucial factors in directing appropriate phenotypic differentiation. Failure can lead not only to a lack of benefit but also to significant side-effects from proliferation of non-neuronal phenotypes.

All these problems can be solved, but it will require more than a single breakthrough to transform the potential attributed to stem cells into a realistic clinical strategy for cellular repair.

what neural cell types they produce; this still remains to be done. Emerging methods for the prospective isolation of adult CNS stem cells[11–14] should facilitate this important experiment. Experiments using culture-expanded adult CNS stem cells indicate that there is some plasticity in adult environments (Table 1). For example, adult spinal-cord-derived stem cells, which do not normally make neurons, can make interneurons if injected into the adult hippocampus[41], and adult hippocampal-derived stem cells can make olfactory interneurons after transplantation to the SVZ[42]. As yet, however, there is no direct evidence that adult-derived stem cells can make the types of projection neuron that are normally generated in the embryo.

Given the vital gaps in our understanding of adult neural stem cells, we cannot yet conclude that they are highly plastic. Evidence that they can generate different somatic cell types is limited, and may be restricted to rare events or rare cells[43–45]. In considering the two different ideas raised at the beginning of this review, there may be in fact no dichotomy. Most neural stem cells might be regionally and temporally specified. There may also be rare stem cells present in the nervous system, perhaps not even of neural origin, that have greater plasticity, at least in terms of producing diverse somatic cell types[43].

Reversing the stem cell lineage

If stem cells are restricted, can these restrictions be reversed (Fig. 4)? For example, can an adult stem cell re-acquire the ability to generate cell types normally made in the embryo? A study has indicated that culturing SVZ stem cells as neurospheres expands their potential and allows them to generate PNS progeny after injection into chick neural crest pathways[46]. Furthermore, descendants of neural stem cells may be able to revert. Optic nerve O2A progenitor cells, which normally produce solely glia, can be converted *in vitro* into multipotent, neurosphere-generating stem cells[47]. In the adult SVZ, both type B stem cells and their progeny, type C progenitor cells, can make neurospheres *in vitro*. Moreover, type C cells are stimulated to convert to stem cells by epidermal growth factor[48]. In the postnatal chick retina after damage, Muller radial glia, which are maintained throughout life, can re-enter the cell cycle, re-express retinal progenitor cell markers and generate new neurons and glia[49].

If environmental factors can enhance the acquisition of neural stem cell fates, or increase the plasticity of stem cells, this may be of enormous benefit therapeutically, as indicated in Box 1.

Future studies

As regions of the embryo are patterned and development unfolds, neural stem cells may be an essential mediator of developmental signals, acquiring a changing repertoire of gene expression, morphology and behaviour. Despite differences in the properties of stem cells isolated from different regions and at different times, they still self-renew. Self-renewal can therefore be considered as the propagation of stem cells, rather than the production of exactly the same type of cell.

It will be important to examine how developmental signals, both spatial and temporal, specify changes in neural stem cells. Markers for neural stem cells will allow their selection from different stages and regions to examine their potential after transplantation into the embryo or adult, and a comparison of their gene expression. Such explorations will help identify essential mediators of stem cell self-renewal, and genes that determine production of different types of progeny. Markers will also help solve the tantalizing issue of which cells *in vivo* are stem cells.

Although research to identify adult sources of highly plastic stem cells for therapeutic use will continue, it seems likely that most neural-generating stem cells might be specified during development. In that case, we must explore this diversity to understand how different neural cells are, and can be, made. □

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EXHIBIT G

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In Vitro Expansion of a Multipotent Population of Human Neural Progenitor Cells

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The isolation and expansion of human neural progenitor cells have important potential clinical applications, because these cells may be used as graft material in cell therapies to regenerate tissue and/or function in patients with central nervous system (CNS) disorders. This paper describes a continuously dividing multipotent population of progenitor cells in the human embryonic forebrain that can be propagated *in vitro*. These cells can be maintained and expanded using a serum-free defined medium containing basic fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF), and epidermal growth factor (EGF). Using these three factors, the cell cultures expand and remain multipotent for at least 1 year *in vitro*. This period of expansion results in a 10^7-fold increase of this heterogeneous population of cells. Upon differentiation, they form neurons, astrocytes, and oligodendrocytes, the three main phenotypes in the CNS. Moreover, GABA-immunoreactive and tyrosine hydroxylase-immunoreactive neurons can be identified. These results demonstrate the feasibility of long-term *in vitro* expansion of human neural progenitor cells. The advantages of such a population of neural precursors for allogeneic transplantation include the ability to provide an expandable, well-characterized, defined cell source which can form specific neuronal or glial subtypes. © 1999 Academic Press

Key Words: **stem cell; development; embryonic; cell culture; progenitor; human; central nervous system.**

INTRODUCTION

Historically, the adult mammalian central nervous system has been considered incapable of regeneration. This conclusion was based, in part, on evidence that neurogenesis is complete shortly after birth (reviewed in 34). However, more recent data indicate that limited neurogenesis occurs in isolated regions of the normal adult nervous system. For instance, neuronal progenitors in the rat dentate gyrus have been found to proliferate in both late postnatal development and in the adult (1, 2, 4). A population of proliferating neural progenitor cells has also been identified in the subependyma of the adult rodent (13, 16, 20, 21). While these findings suggested that specialized populations of cells are able to proliferate and differentiate into neurons throughout an animal's lifetime, proof of a multipotent stem cell in the mammalian CNS remained elusive.

More recently, several laboratories have isolated and expanded rodent progenitor cells in the presence of EGF and/or bFGF (22, 26–28, 31). In these culture systems, removal of mitogens and the addition of serum will result in the differentiation of the progenitor cells into neurons and glia. In 1992 Reynolds and Weiss identified a population of cells in the mouse embryonic and adult CNS that could be cultured under defined conditions and could be induced to differentiate into neurons, astrocytes, and oligodendrocytes, the three main phenotypes in the CNS (26–28). These multipotent cells are maintained as nonadherent clusters, in the presence of the mitogen epidermal growth factor (EGF). These clusters can be expanded in culture for extended periods of time (7, 26, 27), and clonal analysis demonstrates their ability to self-renew and to differentiate into the major neural phenotypes (27), suggesting that these cells represent a neural stem cell population. In other work, a population of neural stem cells has been generated from the adult rat hippocampus. These cells are maintained as an adherent population in the presence of bFGF (22, 25). Although both of the cell populations expand *in vitro* and are multipotent, it is unclear whether these cells are equivalent populations. These mitogen-driven cell culture systems provide a means to exponentially expand a multipotent popula-

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tion of cells and may provide an ideal source of cells for CNS transplantation. However, while rodent neural stem/progenitor cells have been identified in these systems, attempts to expand human neural progenitor cells have not been successful. Other groups have generated human cell lines by derivation from a human teratocarcinoma (33) or by utilizing *v-myc* to drive cell proliferation (29), but these cells do not have the potential to produce the three major neural cell types.

In the experiments presented here, continuously proliferating neural progenitor cell cultures were established from first-trimester human embryonic forebrain. These cultures were maintained in the presence of human basic fibroblast growth factor (hbFGF), human leukemia inhibitory factor (hLIF), and human epidermal growth factor (hEGF). These cultures represent a heterogeneous population of cells which expand exponentially and, upon differentiation, have the potential to form the three major phenotypes in the CNS: neurons, astrocytes, and oligodendrocytes. Under certain differentiation protocols, specific neuronal subtypes can be induced, including cells immunoreactive for tyrosine hydroxylase (TH) and gamma-amino-butyric acid (GABA).

METHODS

Isolation of Tissue

Embryonic brain tissue from 5.0 to 11.0 weeks of gestation was acquired under compliance with NIH guidelines, Swedish government guidelines, and the local ethics committee and appropriate consent forms were used. Samples were collected and the forebrain was dissected and placed into sterile saline. The tissue was transferred to growth medium (see below) and was mechanically dissociated using a standard glass homogenizer. The dissociated cells were seeded into growth medium containing hEGF and hbFGF (see below) at approximately 100,000 cells/ml.

Cell Culture

Cell suspensions were grown in N2, a defined DMEM: F12-based (GIBCO) medium supplemented with 0.6% glucose, 25μg/ml human insulin, 100μg/ml human transferrin, 20 nM progesterone, 60μM putrescine dihydrochloride, 30 nM sodium selenite, 2 mM glutamine, 3 mM sodium bicarbonate, 5 mM Hepes, and 2μg/ml heparin (Sigma). This medium was supplemented with 20 ng/ml hEGF (GIBCO), 20 ng/ml hbFGF (GIBCO), and 10 ng/ml hLIF (R & D Systems). Typically, the cells grew as clusters which were passaged by mechanical dissociation approximately once every 7–30 days (depending on the mitogens used) and reseeded at approximately 75,000–100,000 cells/ml. Cell lines generated in this fashion were designated by gestational age and anatomical region, e.g., forebrain from 5 weeks of gestation was designated 5FBr.

In initial experiments, the progenitor cells were differentiated by plating on poly-ornithine-coated glass coverslips. To induce differentiation, growth factors were removed from the growth medium and the medium was supplemented with 1% FBS (GIBCO). Cells were cultured for 12–14 days before fixation for immunocytochemistry.

In some experiments, cells were differentiated in the presence of cytokines. In these experiments cells were plated onto poly-ornithine-coated coverslips at 50,000 cells/coverslip in N2 supplemented with either 400 pg/ml IL-1b (R & D Systems) or a cocktail of IL-1b (200 pg/ml), IL-11 (1 ng/ml) (R & D Systems), and GDNF (1 ng/ml) (Promega). The cells were allowed to differentiate for 20 days before fixation and immunostaining. Cultures were fed three times each week with fresh media and cytokines.

Immunocytochemistry

The cell cultures were fixed for 10–20 min at room temperature with 4% paraformaldehyde in PBS, washed three times in 0.1 M PBS, pH 7.4, permeabilized using a 2-min incubation in 100% EtOH, and washed again with 0.1 M PBS. Cultures were then incubated in 5% NGS (normal goat serum) in 0.1 M PBS with 0.1% Triton X-100 (Sigma) for at least 1 h at room temperature. Blocking was followed by incubation in primary antibodies diluted in 1% NGS + 0.1% Triton X-100 for at least 2 h at room temperature. The cultures were washed in PBS and incubated with secondary antibodies diluted in 1% NGS with 1% Triton X-100 for 30 min at room temperature in the dark.

The primary antibodies used were human specific nestin, 1:100 (generously provided by U. Lendahl, Karolinska Institute); β-tubulin, 1:1000 (Sigma); glial fibrillary acidic protein (GFAP), 1:500 (DAKO); GABA 1:200 (Sigma); BrdU, 1:1000 (Amersham); galactocerebroside (GalC), 1:25 (Boehringer); and TH, 1:200 (Chemicon). The secondary antibodies used in these experiments were goat anti-mouse FITC, 1:128 (Sigma), and goat anti-rabbit TRITC, 1:100 (Cappel).

Quantitation of the different phenotypes was accomplished by counting immunolabeled cells on each coverslip. For each condition, three to six coverslips were evaluated. Four separate randomly chosen fields were counted on each coverslip using a 40× objective. The number of nuclei was counted using DAPI staining and then the number of β-tubulin-, GFAP-, or GABA-immunoreactive (ir) cells in each field was counted. These numbers were then summed to give a total representative count for each coverslip. A percentage for each phenotype was generated and these numbers were used to generate mean values for each condition.

Statistical analyses included Student's *t* test using StatView software.

RESULTS

Generation of Continuously Proliferating Human Progenitor Cell Cultures

In this paper, the term "cell line" refers to a cell line which is a continuously proliferating population of cells derived from a single embryonic source, where proliferation is induced by mitogens, rather than by oncogenes or transformation. Cell lines (cultures) were derived from nine samples of forebrain tissue from human embryos at 5, 6.5, 7, 8.5 (two separate samples), 9, 9.5 (two separate samples), and 10.5 weeks of gestation. Each cell line was designated by gestational age and anatomical region of the tissue of origin, i.e., "5FBr" is the cell line obtained from embryonic forebrain at 5 weeks of gestation. Two parallel cultures—with and without hLIF—were generated from each of these samples, for a total of 18 cell lines. One of these cell lines (5FBr without hLIF) failed at passage 2, but all other cultures continued to expand, some for as many as 35 passages, and were able to differentiate into neurons, astrocytes, and oligodendrocytes.

Cells derived from the dissociation of embryonic forebrain samples (described under Methods) were suspended in growth medium. In all cultures, many single cells attached to the flasks; however, as the cells proliferated into small clusters, they detached from the plastic and floated in suspension. The clusters continued to increase in size during a period of 7–30 days, depending on growth conditions (see below). After clusters formed, the cells were passaged by mechanical dissociation into a single cell suspension. To date, the cells have been reseeded and carried continuously for up to 17–35 passages (175–371 days), and the cell lines remain viable. Figure 1A illustrates representative clusters from cell line 9FBr at passage 6 (all cell lines appeared similar in their undifferentiated state). The spheres showed positive immunoreactivity to the neuroepithelial stem cell marker, nestin (17) (Fig. 1B), and incorporated bromodeoxyuridine (BrdU) (Fig. 1C), characteristic of actively proliferating cell populations. To date, no consistent difference in growth rate between cultures generated from tissues of different gestational age while maintained in hEGF, hbFGF, and hLIF have been observed (Fig. 1D).

Expansion: Effect of hLIF

In addition to the cultures maintained in hEGF, hbFGF, and hLIF, parallel cultures were generated by placing some of the cells into medium containing only hEGF and hbFGF. Initially, cell lines maintained in hEGF and hbFGF expanded at a rate similar to those cultured with hEGF, hbFGF, and hLIF. However, after about 50–60 days *in vitro*, the cultures without hLIF consistently showed slower expansion; conversely, the cultures that contained all three growth factors continued to expand and their growth rate appeared to increase, the population doubling every 7–10 days (Figs. 2A and 2B). To determine if hLIF would affect cultures which had been continuously maintained in only hEGF and hbFGF, hLIF was added to the $9FBr_{021997}$ cell line which had been maintained only in hEGF and hbFGF for 10 passages. The expansion of these cells was monitored for six more passages. The addition of hLIF resulted in an increase in the proliferation rate of this cell line (Fig. 3A) indicating that at least some cell lines remain responsive to LIF even when maintained in the absence of this cytokine.

LIF is a member of the IL-6 cytokine family which also includes oncostatin M, IL-11, ciliary neurotrophic factor (CNTF), and Flt3/Flk2. Other members of this cytokine family were tested to determine if this growth effect was unique to LIF. Preliminary experiments indicate that cells grown in hEGF, hbFGF, and hCNTF (10 ng/ml) show a proliferation rate similar to cells grown in hEGF, hbFGF, and hLIF. However, if hLIF is replaced with hFlt3/Flk2 (10 ng/ml) or IL-6 (10 ng/ml) the growth rate is reduced (data not shown).

Expansion: Effect of bFGF

To determine if hbFGF was essential for cell proliferation, hbFGF was removed from the $9FBr_{021997}$ and $5FBr_{072397}$ cell lines, which had been previously maintained in hEGF, hbFGF, and hLIF (as described above), and the growth rates of cells under the two culture conditions were compared. Proliferation of both cell lines declined immediately upon removal of hbFGF, although cells remained viable in the absence of this growth factor. The results for the $9FBr_{021997}$ cell line are shown in Fig. 3B. The $9FBr_{021997}$ cell line has been maintained in the absence of hbFGF for as many as 22 passages (230 days), and multipotency has been evaluated (see below). These results indicate that hbFGF, similar to hLIF, appears to promote proliferation of these cells.

Expansion: Effect of EGF

To assess whether hEGF was necessary for proliferation of the human cells, hEGF was removed from the $5FBr_{072397}$ and $8.5FBr_{032598}$ cell lines. As shown in Fig. 3C, the initial growth rates of both cell lines did not appear to be affected by the removal of hEGF. It is unlikely that this is due to the absence of the EGF receptor because RT-PCR data for these cell lines showed the presence of mRNA for the human EGF receptor (data not shown).

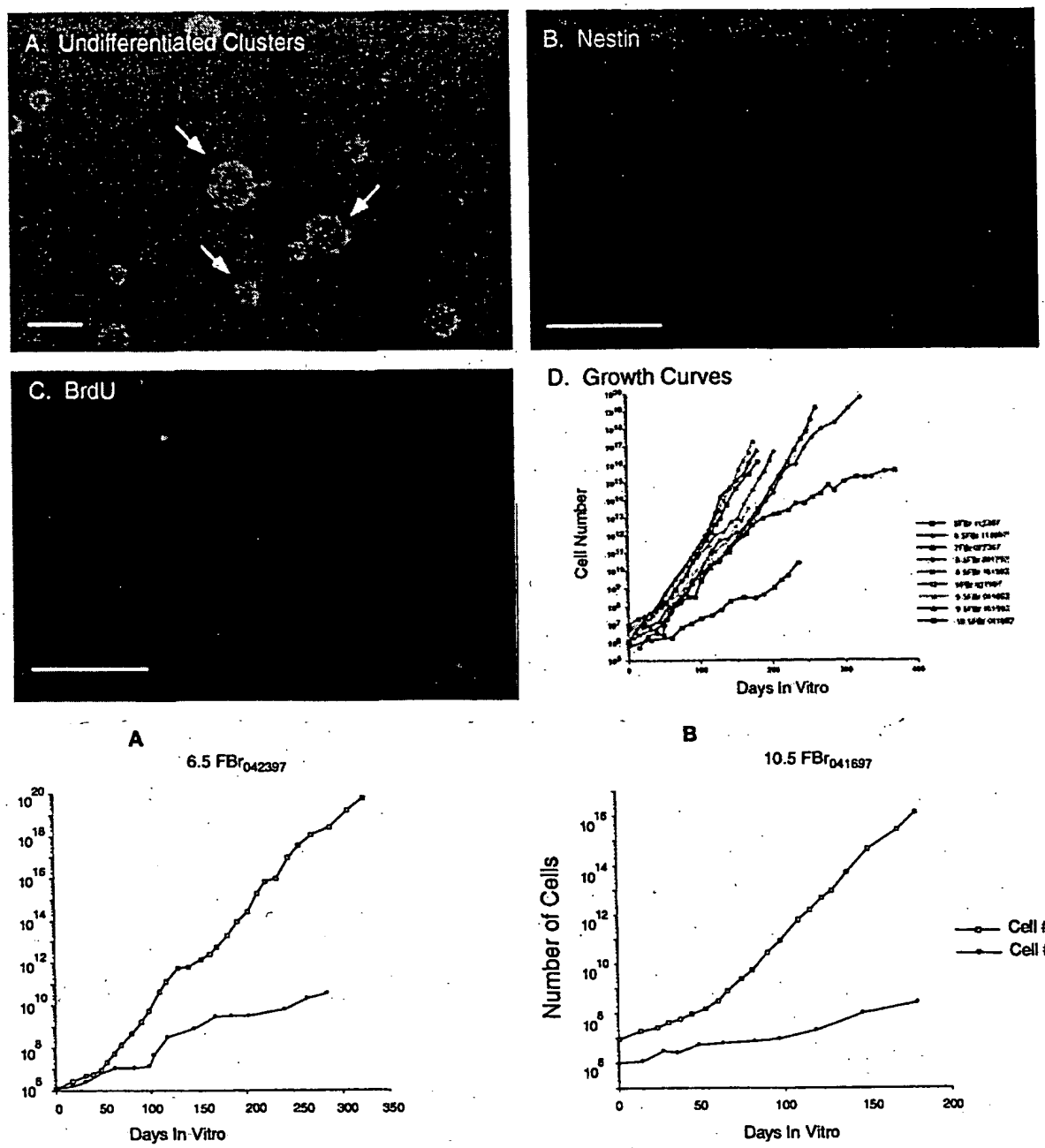
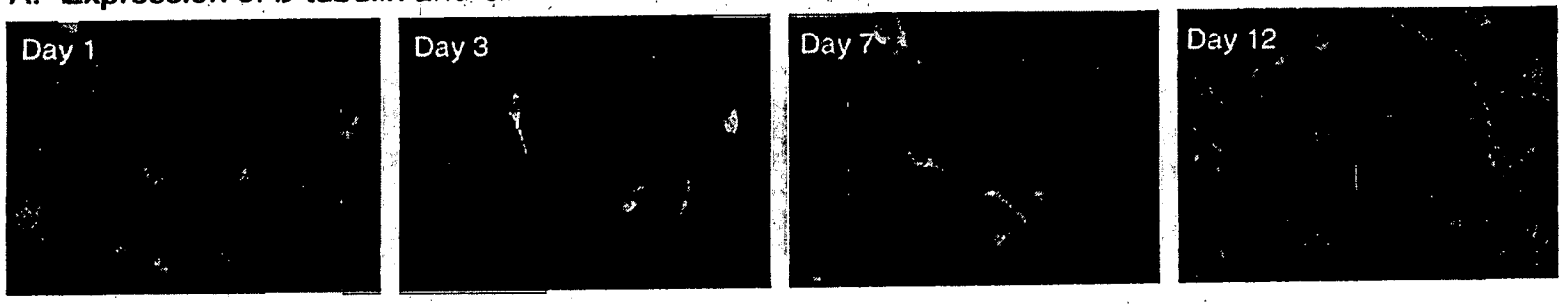


FIG. 1. Human neural progenitor cells. (A) Phase-contrast photograph of undifferentiated human neural progenitor cells ($9FBr_{021997}$) after six passages. Arrows indicate clusters of cells in suspension. (B) Nestin immunoreactivity in undifferentiated neural progenitor cell clusters. Because these cells are very tightly packed, the nestin immunoreactivity does not appear filamentous. In all experiments control cultures were included which received only the secondary antibody. These controls did not show any positive staining. (C) BrdU incorporation in undifferentiated neural progenitor cell clusters. Scale bars in all images represent 100μm. (D) Growth rates for nine "cell lines" maintained in hEGF, hbFGF, and hLIF.

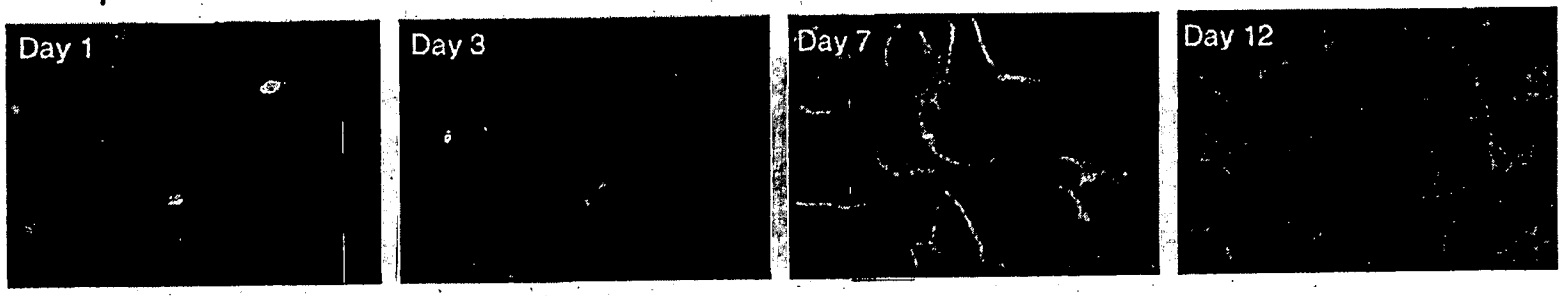
FIG. 2. Growth rates of human neural progenitor cells. Parallel cultures were maintained in hEGF and hbFGF or in hEGF, hbFGF, and hLIF. Cultures were generated from forebrain at gestational weeks 6.5 and 10.5. Cell lines maintained in the absence of hLIF showed slower proliferation rates than cell lines maintained in hLIF.

FIG. 4. Characterization of human progenitor cells during differentiation *in vitro*. Cells were plated on day 0 and were fixed and stained on days 1, 3, 5, 7, and 12. (A) β-Tubulin and GFAP expression increased with time, until about 1 week *in vitro*. In these images, fluorescein represents β-tubulin-ir and Texas red represents nestin-ir. (B) Nestin expression decreased with time *in vitro*. In these images, fluorescein represents β-tubulin-ir and Texas red represents nestin-ir. (C) Quantification of the expression of β-tubulin, GFAP, and nestin indicates that while nestin expression decreases over time, both β-tubulin and GFAP expression increase.

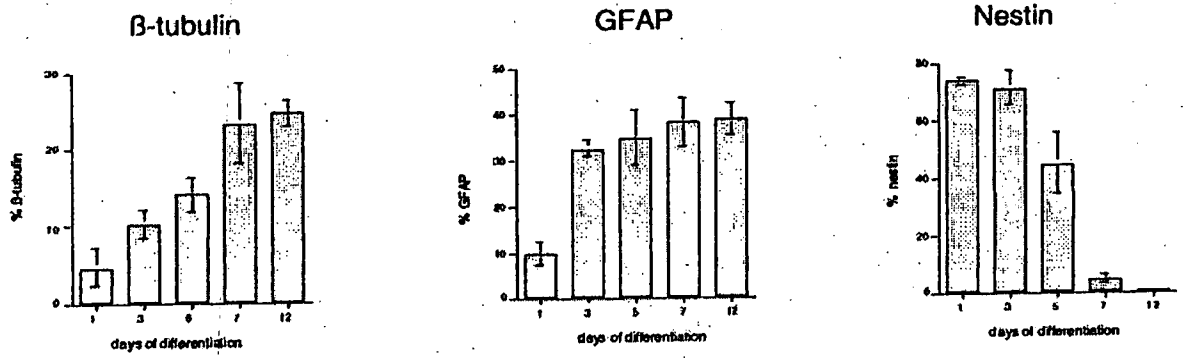
A. Expression of ß-tubulin and GFAP



B. Expression of ß-tubulin and nestin



C. Expression of Phenotypic Markers During Differentiation



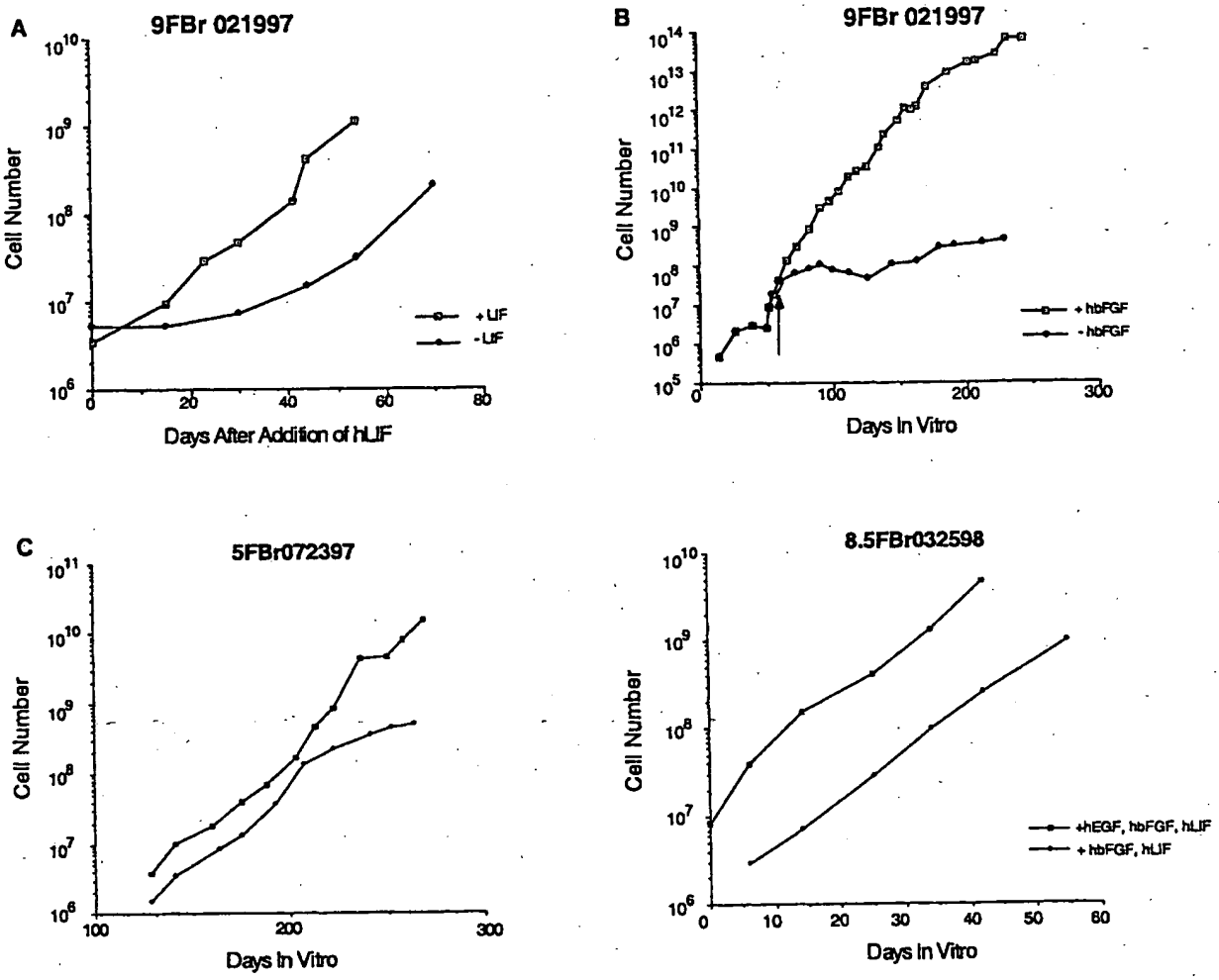


FIG. 3. The effects of hLIF, hbFGF, and hEGF on growth rates. (A) The $9FBr_{021997}$ cell line was maintained in hEGF and hbFGF for 10 passages before hLIF was added. In this graph, day 0 represents the initial addition of hLIF at P10. The addition of hLIF resulted in an immediate increase in growth rate. (B) The removal of hbFGF from the 9FBr cell line resulted in an immediate decrease in growth rate. The removal of hbFGF is indicated by the arrow. (C) Two cell lines ($5FBr_{072397}$ and $8.5FBr_{032598}$) were maintained in the absence of hEGF for 5–20 passages. No difference was seen in the growth rate of the cultures in which EGF was removed.

To date, human progenitor cell lines have been carried for as long as 35 passages in hLIF ($9FBr_{021997}$ for 371 days) and for 22 passages without hLIF ($9FBr_{021997}$ for 211 days). All cell lines continue to expand and form spheres.

Differentiation: General Observations

Unless otherwise specified, the cells were differentiated by plating onto poly-ornithine-coated glass coverslips in N2 medium supplemented with 1% FBS. Under these conditions, the cells immediately adhered to the substrate and began to differentiate, and after several days *in vitro*, different morphologies were apparent, including large flat cells and small bipolar and multipolar cells. Within 7 to 10 days the cells had proliferated to create a monolayer. Three cell lines were evaluated on days 1, 3, 5, 7, and 12 for expression of β-tubulin, GFAP, and nestin. All three displayed a similar capacity to differentiate and representative data from one of these cell lines are presented in Fig. 4. On day 1 of differentiation only a few cells were β-tubulin-immunoreactive (β-tubulin-ir) or GFAP-immunoreactive (GFAP-ir) (Figs. 4A and 4C). The percentage of β-tubulin-ir and GFAP-ir cells increased over time and seemed to reach a plateau at about 7 days (Fig. 4C). In contrast, the expression of nestin decreased over time after induction of differentiation (Figs. 4B and 4C). On day 1, about 75% of the cells were nestin-ir which declined to less than 5% within 1 week.

Cell lines generated from different gestational ages

were allowed to differentiate for 12–14 days and were evaluated by immunocytochemistry for expression of specific phenotypic markers. As seen in Figs. 5A and 5B the cells expressed GFAP, GalC and β-tubulin III, indicating the presence of glia and neurons. Each cell line showed different percentages of neurons and astrocytes. The percentage of GFAP-ir cells ranged between 15 and 53% in different cell lines. Morphologically, some GFAP-positive cells were large and flat, resembling type 1 astrocytes (Fig. 5A); others exhibited a more stellate morphology, similar to that of type 2 astrocytes (data not shown). Neither the variation in numbers nor the morphology appeared to correlate with the gestational age of the tissue used to generate the cell line. Evaluation of GFAP-ir cells was difficult in some cases where the cells became so confluent that cell boundaries were often obscured, creating an obstacle to accurate cell counting. These cases were therefore excluded from cell counts and data analysis.

The number of neurons was assessed by counting the cells that demonstrated immunoreactivity to β-tubulin isotype III, a marker for neurons *in situ* and *in vitro* (3, 5, 9, 11). All of the cell lines generated neurons; however, different cell lines generated different percentages (20–37%) of β-tubulin III-positive neurons (Table 1). Many neurons showed elaborate arborizations, but markers for more mature neurons (such as neurofilament or NSE) were not evaluated in these studies. However, these cells demonstrated positive immunoreactivity to human neurofilament antibodies after transplantation into the adult rat brain, indicating their capacity to form mature neurons (12). In all cell lines assessed *in vitro*, a number of neurons showed immunoreactivity to GABA (GABA-ir) (Figs. 5C and 5D) which colocalized with β-tubulin III (Fig. 5D). The number of GABA-ergic neurons varied (9–51% of neurons) between cell lines generated from different gestational ages, but no developmental trends were seen in these data (Table 1).

Using an induction protocol recently described by Ling *et al.* (1998), immunoreactivity to TH was observed (Fig. 5F). This protocol uses a combination of IL-1b (200 pg/ml), IL-11(1 ng/ml), and GDNF (1 ng/ml) to induce the formation of TH-ir cells. In the experiments presented here, cells differentiated in this cocktail of factors or differentiated in IL-1b (400 pg/ml) alone formed TH-ir cells after 20 days *in vitro*. At this time, small patches of cells were identified which were TH-ir. In addition, these cells colocalized β-tubulin, indicating neuronal expression of TH. These data were supported by positive RT-PCR results indicating the presence of mRNA for human TH (data not shown). Because these cells were found in patches, it was difficult to accurately determine the percentage of the population which became TH-ir. This finding is currently being further characterized.

Differentiation: Effect of hLIF, hbFGF, and hEGF

As described above, cell lines maintained with and without hLIF exhibited very different growth rates. The multipotency of these parallel cultures was also investigated. Four cell lines ($6.5FBr_{042397}$, $7\ FBr_{072397}$, $8.5FBr_{091797}$, and $9FBr_{021997}$) were induced to differentiate following the standard differentiation protocol described above, and the number of neurons formed was assessed. Cells cultured in hLIF consistently produced significantly more neurons than cells cultured without hLIF ($P < 0.001$ and $P < 0.0001$) (Fig. 6A). Furthermore, qualitatively, the neurons generated in the cultures carried in hLIF before differentiation appeared to have more elaborate processes (data not shown).

Cells maintained in the presence of hbFGF produced significantly more neurons than parallel cultures maintained without hbFGF. As described previously, hbFGF was removed from the $9FBr_{021997}$ cell line, resulting in a decrease in proliferation. As shown in Fig. 6B, the number of neurons formed upon differentiation was dramatically reduced after hbFGF removal ($P < 0.05$). In addition, the neurons which were observed had short processes resembling immature neurons (Fig. 5E).

The presence or absence of hEGF did not appear to affect the differentiation of neurons or astrocytes. hEGF was removed from the $5FBr_{072397}$ and $8.5FBr_{032598}$ cell lines for five passages. In the $5FBr_{072397}$ cell line, hEGF was removed at passage 10 and the cells were evaluated for multipotency at one and five passages after this change. At both time points, the percentage of neurons remained similar ($5FBr_{072397}$ shown in Fig. 6C). The percentage of astrocytes appeared to drop after five passages without hEGF. hEGF was also removed from the $8.5FBr_{032598}$ cell line at passage 1 and multipotency was evaluated at passage 5. In this cell line, differentiation into neurons and astrocytes did not seem to be affected by the removal of hEGF.

Differentiation: Effect of Long-Term Culture

Differentiation of three of the cell lines ($6.5FBr_{042397}$ + LIF, $9FBr_{021997}$ + LIF, and $10.5FBr_{041697}$ + LIF) was characterized at various passages (Fig. 7). As mentioned previously, in some cases it was difficult to assess the number of GFAP-ir cells because accurate cell counts could not be obtained at some time points when the cells tended to grow in a monolayer. In cell line $9FBr_{021997}$ the percentage of cells expressing β-tubulin III remained stable (about 20%) until passage 21, and then it decreased by passage 30 to 14% (Fig. 7A). In the other two cell lines, the number of neurons generated decreased with passaging: in line $6.5FBr_{042397}$ they decreased from 37% at passage 5 to 13% at passage 20 (Fig. 7B); and in line $10.5FBr_{041697}$ they decreased from 29% at passage 5 to 12% at passage 20 (Fig. 7C). These

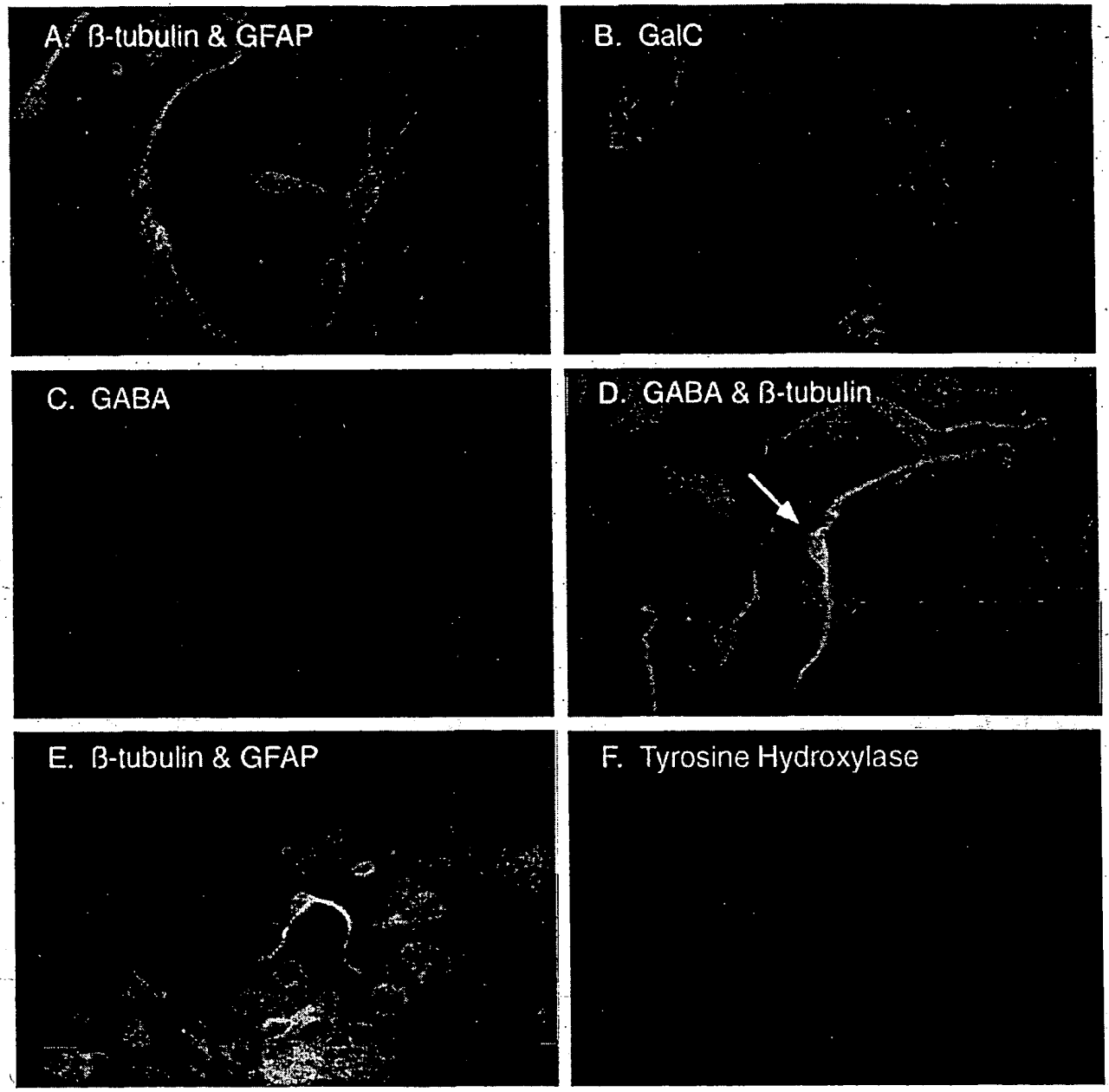


FIG. 5. Trilineage potential of human progenitor cells upon differentiation. (A) $9FBr_{021997}$ cell line maintained in hEGF, hbFGF, and hLIF for 30 passages before differentiation expresses β-tubulin (green) and GFAP (red). (B) GalC immunoreactive cells in the differentiated $9FBr_{0219997}$ cell line maintained in hEGF and hLIF (no hbFGF) for 22 passages. (C) GABA immunoreactive neuron from the $5FBr_{112397}$ cell line maintained in hEGF, hbFGF, and hLIF for 5 passages. (D) GABA (red) and β-tubulin (green) immunoreactivity in differentiated cultures from the $5FBr_{112397}$ cell line maintained in hEGF, hbFGF, and hLIF for 5 passages. One cell is double labeled with both markers (yellow, indicated with arrow). (E) GFAP (red) and β-tubulin (green) immunoreactivity in the $9FBr_{021997}$ cell line maintained in the absence of hbFGF (hEGF and hLIF only) for 22 passages. The β-tubulin cell has short processes indicative of an immature neuron. Cell nuclei are labeled with DAPI (blue). (F) Positive TH immunoreactivity found in human neural progenitor cells differentiated in cytokines for 20 days. The $8.5FBr_{032598}$ cell line was passaged five times in hEGF, hbFGF, and hLIF and then differentiated in N2 + 1% FBS supplemented with IL-1β (200 pg/ml), IL-11 (1 ng/ml), and GDNF (1 ng/ml) for 20 days.

TABLE 1

Quantitation of Multipotentiality of Human Neural Progenitor Cell Lines Generated from Forebrain at Gestational Weeks 6.5, 7, 9, and 10.5

Age of gestation (weeks)	% GFAP	% β-tubulin	% GABA (% of neurons)
6.5 (P5)	15 ± 3	37 ± 3	21 ± 4
7 (P7)	N/A	22 ± 1	9 ± 3
9 (P7)	53 ± 4	20 ± 0.4	35
10.5 (P5)	50	29 ± 2	51 ± 5

Note. Cell lines were carried for five to seven passages before quantification. The percentage of cells expressing GFAP and β-tubulin was evaluated. The percentage of neurons expressing GABA is also shown. Data are presented as mean ± SEM.

data may be indicative of shifting ratios of progenitor cell populations in these cultures.

All of the cell lines evaluated produced oligodendrocytes, as indicated by immunoreactivity to GalC. GalC-ir cells were typically found in patches and the overall number GalC-ir cells was generally quite low and varied between cell lines. Because the distribution of these cells was sparse and uneven, it was difficult to accurately quantify the percentage of cells which formed oligodendrocytes. GalC-ir cells were identified in cell lines maintained in hEGF, hbFGF, and hLIF as well as in hEGF and hLIF. Although the presence/absence of different mitogens affected the number of neurons formed, it is unclear if the mitogen cocktail affects the number of oligodendrocytes formed. Figure 5B illustrates GalC-ir in the $9FBr_{021997}$ cell line after 22 passages.

DISCUSSION

The ideal type of cells for CNS transplantation will be an expandable population of cells which can differentiate into appropriate phenotypes. The cell culture system described here meets these criteria. Other human cell lines exist that may be used for transplantation. Both the human teratocarcinoma cell line (NT2) (33) and the conditionally immortalized tetracycline-responsive v-*myc* cell line (29) can be expanded and induced to form postmitotic neurons; however, these cell lines do not form all three neural phenotypes. More recently, human neural stem cells have been derived using v-*myc* which can be expanded for long periods *in vitro* (10). It is unclear whether the proliferation rate of these cells is similar to that of the cells described in this report.

The data presented here indicate that a population of human neural progenitor cells exist in the first trimester which can be expanded *in vitro* and have the capacity to differentiate into neurons, astrocytes, and oligodendrocytes. This expansion is dependent upon mitogens and does not require genetic modification of the cells. The greatest expansion of cells is observed using hEGF, hbFGF, and hLIF as growth factors in the medium. Although EGF and bFGF have been used in a variety of progenitor and stem cell culture systems, the use of LIF in this culture system appears to be unique. These cultures contain a mixture of progenitor cell types with various differentiation potentials. It is likely that each of the mitogen conditions which were investigated affected subpopulations of progenitor cells differently, resulting in the observed variations in multipotency. This conclusion is most clearly illustrated by the differences seen in neuronal formation in cultures maintained with and without hbFGF and hLIF. Although the described cultures proliferate and remain multipotential for extended periods of time in the three mitogens, it is not clear that this is the optimal growth condition for these cells. Upon removal of EGF, two cell lines continued to proliferate and maintained their multipotency for at least five passages. The requirement of EGF and different media and mitogen cocktails is currently being evaluated to determine the appropriate growth conditions for the progenitor cultures.

It is of particular interest that cultures maintained in the presence of LIF consistently produced more neurons than parallel cultures carried in the absence of LIF. LIF is known to be an essential factor in the maintenance of embryonic stem cell (ES) cultures. While the action of LIF in ES cultures is believed to be via the inhibition of differentiation (23, 30, 32), it is not clear what the action of LIF is in the human neural progenitor cell cultures presented in this study. It is possible that LIF is acting to inhibit the differentiation of cells such that the multipotent nature of these cells is maintained for longer periods of time. However, it is also possible that LIF is selectively supporting the survival of a population(s) of neural progenitor cells. Neuronal formation was found in all nine cell lines grown in the presence of hEGF, hbFGF, and hLIF. In fact, the percentage of neurons (20–37%) observed in these cultures was considerably greater than that seen in murine cultures (5–7%). To further examine this finding, both rat and mouse neurosphere cultures were grown in this mixture of mitogens. However, these cultures failed within two passages (M. K. Carpenter, unpublished observations) indicating that the population of cells isolated from rodent and human sources may be different populations of cells.

The effect of LIF in enhancing growth rate was not evident until 50–60 days in culture. The mechanism for this delayed effect is unclear. It is possible that these first 50–60 days in culture represent the typical life span of one population of progenitor cells which is responsive to LIF, and after this initial period the cells maintained in the absence of LIF die. Alternatively, this may be due to a change in the ratio of progenitor

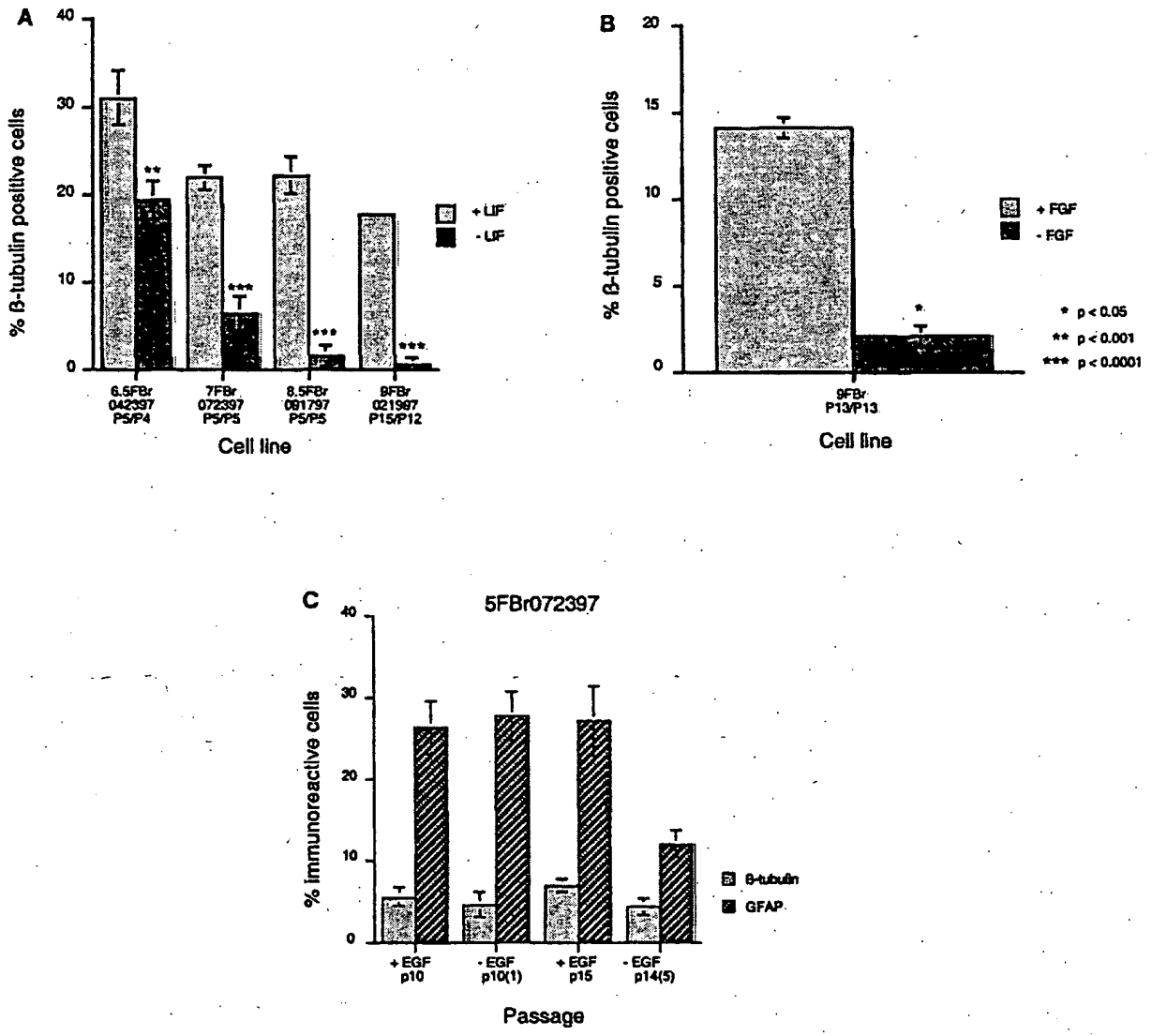


FIG. 6. Effect of mitogens on neuronal and glial differentiation. (A) Neuronal formation was significantly greater in cultures maintained in hEGF, hbFGF, and hLIF than in parallel cultures maintained in only hEGF and hbFGF. Cultures generated from forebrain at gestational weeks 6.5, 7, 8.5, and 9 were evaluated. Parallel cultures were evaluated at similar passages (the specific passage numbers for each cell line are indicated with the cell line designation), indicated by each bar. $^{**}P < 0.001$ and $^{***}P < 0.0001$. (B) Neuronal formation was significantly greater in cultures maintained in hEGF, hbFGF, and hLIF than in cultures maintained in hEGF and hLIF. The $9FBr_{021997}$ line was evaluated at P13 with and without hbFGF as indicated by each bar. $^{*}P < 0.05$. (C) Removal of hEGF did not appear to affect the formation of either neurons or astrocytes. The $5FBr_{072397}$ cell line was evaluated at one and five passages after EGF removal (at P10). For all graphs, error bars indicate SEM.

cells in the cultures or to the temporal expression of mitogen receptors on the cells.

LIF is a member of the gp130 signaling family, which signals through shared components of their multisubunit receptors. The effects of the other family members on the proliferation and differentiation of the human neural progenitor cells are currently being evaluated to determine whether additional mitogens will influence the characteristics of these cultures. CNTF is known to have many effects on very early neuronal precursors (17) and also maintains the pluripotentiality of ES cells (8). Our preliminary data indicate that CNTF supports the expansion of the population of cells. Although CNTF and LIF appear to have a similar effect on cell expansion, neither Flt3/Flk2 nor IL-6 appears to support cell expansion. These factors

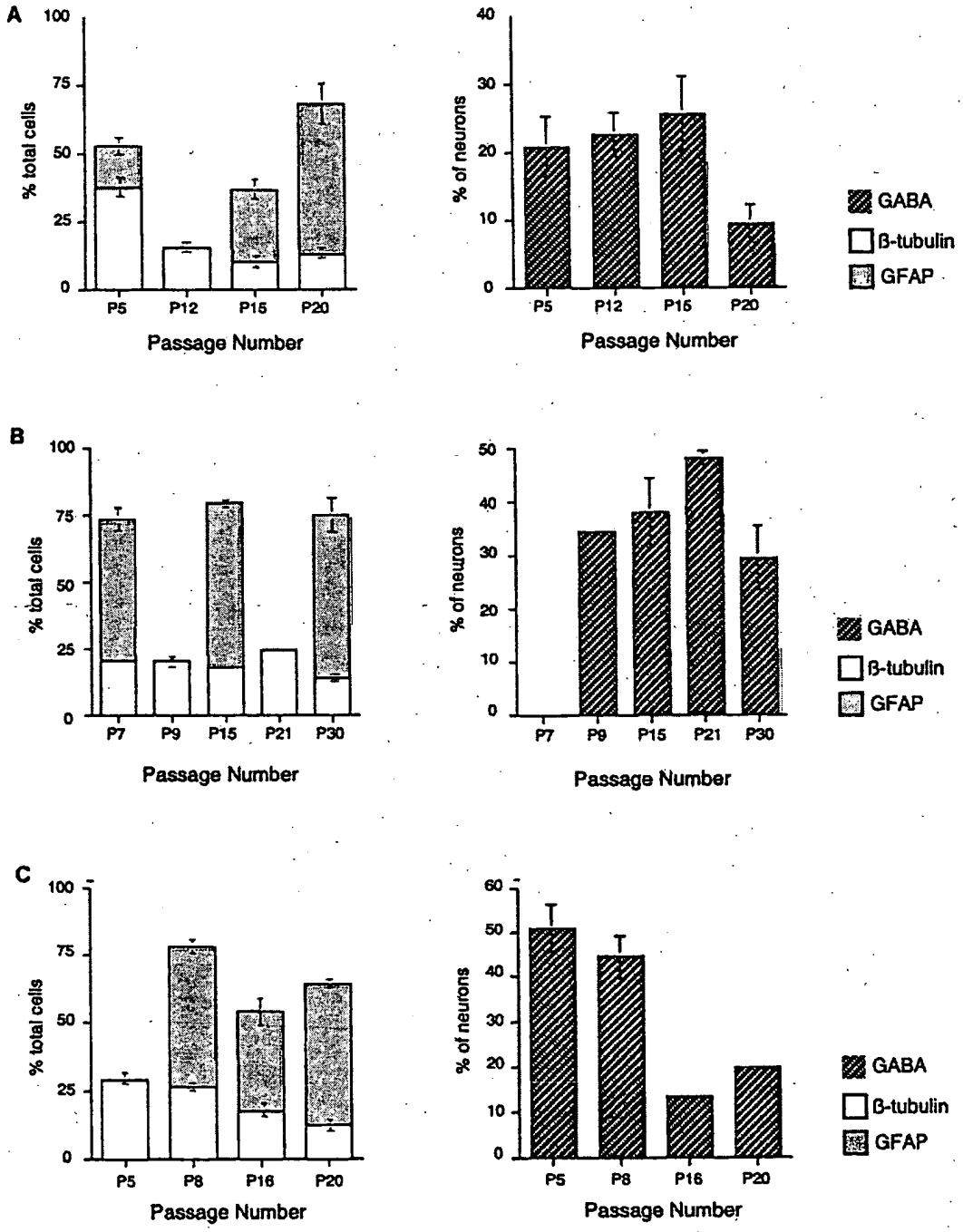


FIG. 7. The effect of long-term culture on the multipotentiality of neural progenitor cells. The percentages of neurons and astrocytes produced by the 6.5 FBr_{042397} (A), $9FBr_{021997}$ (B), and $10.5FBr_{041697}$ (C) cell lines were evaluated at different passages. Note that for cell line $9FBr_{021997}$, passages 9 and 21, and cell line 6.5 FBr_{042397}, passage 12, the number of astrocytes could not be accurately quantified and the values were not included. The number of GABA-positive neurons was assessed in each cell line at different passages. In the $6.5FBr_{042397}$ and $10.5FBr_{041697}$ cell lines, the percentage of cells appeared to decline by passage 20.

are currently being characterized for their effects on multipotency.

bFGF is known to have a mitogenic effect on many different progenitor and stem cell culture systems, including mouse and human cells (14, 15, 22). It is therefore not surprising that hbFGF increased the expansion of the human neural progenitor cell lines described here. However, survival of some portion of the population was not dependent on bFGF, since all of the cells did not die when hbFGF was removed. Furthermore, the removal of hbFGF resulted in a dramatic decrease in the formation of neurons, much like the effect of hLIF removal. bFGF has also been shown to have a mitogenic effect on a variety of forebrain progenitor cells (reviewed in 35). In murine cortical stem cells, bFGF regulates the induction of neurons and glia in a concentration-dependent fashion (24). Exposure to low concentrations of bFGF (0.1 ng/ml) results in the formation of neurons, while exposure to higher concentrations (1–10 ng/ml) results in the formation of oligodendrocytes (24). In the studies presented here, removal of hbFGF from the human progenitor cells resulted in a decrease in neuronal formation. However, various levels of hbFGF were not evaluated to determine the optimal concentration of hbFGF for neuron formation. It is unclear whether the removal of hbFGF results in the death of neural precursors or whether this results in other fate choices for these cells.

Both bFGF and LIF appeared to be necessary for the long-term expansion and for maintenance of the multipotency of cell lines. These growth factors appeared to affect the ratios of progenitor cells in the population. Therefore, it may be possible to enrich a population of cells for neuronal progenitors or oligodendrocyte progenitors by altering the growth factors to which the cells are exposed in their undifferentiated state.

It is particularly interesting that the removal of EGF did not affect the growth rate or the formation of neurons. The initial characterization of neurospheres derived from rodents was accomplished using EGF as the only mitogen (26–28). These findings indicate that, although the cell population expresses the mRNA for the EGF receptor (data not shown), EGF may not be necessary for the maintenance of the human progenitor cells. However, long-term survival experiments will be necessary to confirm this finding. Furthermore, it is unclear if the presence or absence of EGF affects the specific neuronal phenotype (GABA-ergic, dopaminergic, etc.) or the ability to form oligodendrocytes.

The data presented here indicate that the cells isolated from the human CNS respond to mitogens differently than the cells isolated from the rodent CNS. This is further supported by the finding that the rodent-derived neurospheres cannot be maintained in the presence of LIF. In addition, murine neurosphere cultures have been previously derived from LIF receptor knockout mice. These cells expanded well in the presence of EGF and generated neurons, astrocytes, and oligodendrocytes (M. K. Carpenter, unpublished observations). Taken together, these data indicate that the mitogen requirements for the expansion of the rodent and human cells may be different.

In all cell lines that underwent the *in vitro* differentiation process there was a population of cells (20–25%) which did not stain positively for any of the markers used, including nestin. Morphologically, these adherent cells appeared flat and often were polygonal. They may represent a population of nestin-negative progenitor cells, which persist under the conditions used to induce differentiation. Previous experiments using murine cultures have demonstrated that a population of tripotential progenitor cells can persist under differentiating conditions (6). It remains to be seen whether this population of cells represents a progenitor cell population, a differentiated population of cells which has not been identified, or a nonneuronal population of cells.

Upon differentiation, GABA-ergic neurons were identified in all of the cell lines examined. Using the standard differentiation protocol TH-ir cells were not identified. However, this population of human neural progenitor cells did show the capacity to differentiate into TH-expressing neurons. The addition of the hematopoietic cytokine IL-1b resulted in the expression of TH which was colocalized with β-tubulin. This is consistent with previous work using rat mesencephalic progenitor cells (19). The expression of TH in murine-derived neurosphere cultures has also been demonstrated using a combination of E-C-L attachment matrix and NGF during the differentiation process (18). These data indicate that, although TH expression is not a "default" fate choice for these progenitor cells, the cells have the capacity to form TH-expressing neurons *in vitro*. Furthermore, upon transplantation into the adult rat subventricular zone or rostral migratory pathway, these human neural progenitor cells have demonstrated the ability to migrate to the olfactory bulb and form TH-positive neurons (12). Therefore, upon exposure to appropriate environmental cues, this population of human neural progenitor cells has the ability to form different subtypes of neurons both *in vitro* and *in vivo*.

In addition to neurons and astrocytes, oligodendrocytes were identified in human neural progenitor cell cultures. Immunoreactivity to GalC was seen in all cell lines. Although proteolipid protein (PLP) immunoreactivity was occasionally identified (data not shown), immunoreactivity to myelin basic protein (MBP) was not identified. It may be necessary to differentiate the cells for longer periods of time to find robust expression of mature oligodendrocyte markers such as PLP and MBP. Alternatively, the environment used for differen-

tiation (1% FBS) may not provide the factors necessary for the survival of mature oligodendrocytes.

Although these data indicate the presence of an expandable, multipotent population of cells, they do not provide definitive proof of the presence of a human neural stem cell. Stem cells are defined as having the capacity for self-renewal. The human cells described here can be passaged and expanded over 1 year. However, clonal analysis of this culture system is necessary to determine if this population contains neural stem cells. Initial attempts at clonal analysis by limiting dilution of these cells have been hindered by their sensitivity to population density.

At the time of this writing, the ultimate capacity for expansion of these cell lines is unknown; however, the minimum expansion period appears to be at least 1 year. These cells have the capacity to form astrocytes, oligodendrocytes, and neurons. Furthermore, the neuronal phenotype of the cells can be influenced by the environment, indicating that these cells will respond to environmental cues. As potential material for replacement therapy in CNS degenerative diseases, this culture system may therefore offer many advantages, including the ability to expand, safety test, and bank these cells before transplantation.

ACKNOWLEDGMENTS

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EXHIBIT H

RESEARCH ARTICLE

Neural progenitors from human embryonic stem cells

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The derivation of neural progenitor cells from human embryonic stem (ES) cells is of value both in the study of early human neurogenesis and in the creation of an unlimited source of donor cells for neural transplantation therapy. Here we report the generation of enriched and expandable preparations of proliferating neural progenitors from human ES cells. The neural progenitors could differentiate *in vitro* into the three neural lineages—astrocytes, oligodendrocytes, and mature neurons. When human neural progenitors were transplanted into the ventricles of newborn mouse brains, they incorporated in large numbers into the host brain parenchyma, demonstrated widespread distribution, and differentiated into progeny of the three neural lineages. The transplanted cells migrated along established brain migratory tracks in the host brain and differentiated in a region-specific manner, indicating that they could respond to local cues and participate in the processes of host brain development. Our observations set the stage for future developments that may allow the use of human ES cells for the treatment of neurological disorders.

ES cell lines are derived from the pluripotent cells of the early embryo[1–3]. ES cell lines can potentially maintain a normal karyotype infinitely on culture *in vitro* and can differentiate into any cell type[4]. ES cell lines have recently been derived from human blastocysts[5,6], and their potential to differentiate into neural lineages has been demonstrated both *in vivo* in teratomas, and *in vitro*[6–8]. The differentiation of human ES cells into neural progeny may serve as an *in vitro* model for the study of early human neurogenesis. Furthermore, it may enable the development of *in vitro* models of human neurodegenerative disorders, the creation of high-throughput screens for the discovery of neuroprotective and neurotoxic agents, and the identification of novel genes, growth and differentiation factors that have a role in neurogenesis. The potential use of human ES cells as a renewable source of neural cells for transplantation and gene therapy[9] also attracts much public attention.

When ES cells are induced to differentiate *in vitro*, they give rise to a mixture of progeny from the three embryonic germ layers[8,10]. However, we require a means to control differentiation of ES cells into a purified neural progenitor cell population to realize many of their potential applications in neuroscience and regenerative medicine in the central nervous system (CNS). In the mouse ES cell system, strategies for the generation of enriched preparations of proliferating neural progenitors have been developed[11,12]. The *in vitro*–generated neural progenitors could differentiate *in vitro* into both glial cells and functional postmitotic neurons[11]. Transplantation experiments have demonstrated the potential of mouse ES cell–derived neural progenitors to participate in brain development[13], to myelinate axons in host brain and spinal cord[14,15], and to promote recovery after spinal cord injury[16].

We have recently demonstrated that human ES cells can also give rise to neural progenitor cells *in vitro*, and have further demonstrated that the progenitors can differentiate *in vitro* into mature neurons[6]. Here, we extend this work, demonstrating the derivation of highly enriched and expandable populations of proliferating neural progenitors from human ES cells. Furthermore, the neural progenitors could differentiate *in vitro* into mature neurons, astrocytes, and oligodendrocytes. When grafted into the brain ventricles of newborn mouse, the human neural progenitors migrated into the host brain and differentiated in a region-specific manner, according to normal developmental cues, into progeny from the three fundamental neural lineages.

Results

Derivation and propagation of progenitor cells from human ES cells. To derive enriched preparations of neural progenitors, differentiation of human ES cells was induced by prolonged culture (three to four weeks) without replacing of the mouse embryonic fibroblast feeder layer[6]. One week after passage, changes in cell morphology could be identified mainly in the center of the colonies, indicating the initiation of early differentiation. At this time, the expression of transcripts of the neuroectodermal markers nestin and PAX-6 was demonstrated by RT-PCR (Fig. 1A). The expression of transcripts of neural markers could reflect either some constant background differentiation or the process of early neural differentiation.

During the next two weeks of culture, the process of differentiation was markedly accelerated, mainly in the center of the colonies, and cells with short processes that expressed the early neuroectodermal marker N-CAM (neural cell adhesion molecule) could be identified[6]. It appeared that the N-CAM$^+$ cells were growing out from adjacent but distinct areas that were composed of small, piled, tightly packed cells that did not react with the monoclonal antibody

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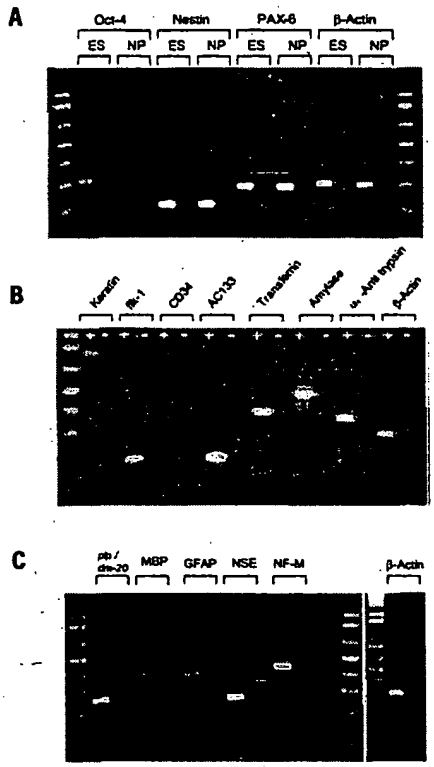

Figure 1. RT-PCR analysis of the expression of markers in human ES cell colonies, ES-derived spheres, and in differentiated cells originating from the spheres. (A) Oct-4, nestin, and PAX-6 in human ES cell colonies at one week after plating and in neural progenitor (NP) spheres. (B) The expression of non-neural marker genes in human ES cell–derived spheres. (C) Neuronal and glial markers in differentiated cells originating from human ES cell–derived neural progenitor spheres. All panels show 2% agarose gels stained with ethidium bromide. The symbols + and – indicate whether the PCR reaction was done with or without the addition of reverse transcriptase. A 1 kb plus DNA ladder was used in all panels. Oct-4 band is 320 bp, nestin 208 bp, PAX-6 274 bp, β-actin 291 bp, keratin 780 bp, Flk-1 199 bp, CD34 200 bp, AC133 200 bp, transferrin 367 bp, amylase 490 bp, α1-antitrypsin 360 bp, *plp* and *dm-20* are 354 bp and 249 bp, respectively, MBP is 379 bp, GFAP is 383 bp, NSE is 254 bp, and NF-M is 430 bp.

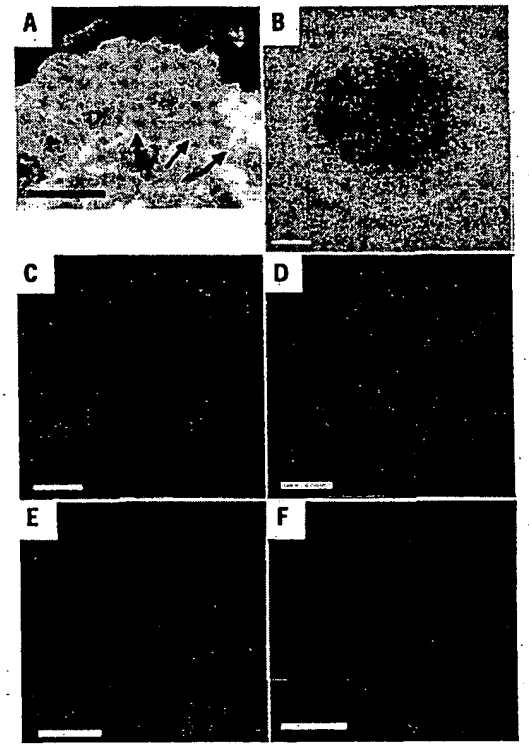

Figure 2. Analysis of morphology and marker expression in human ES-derived progenitor cells. (A) Dark-field stereomicroscopic photograph of a differentiating ES cell colony, four weeks after plating, with areas of cells (arrows) that are destined to give rise to neural progenitors. (B) Phase contrast micrograph of a sphere cultured in serum-free medium. (C–F) Indirect immunofluorescence staining of progenitor cells, 4–12 h after disaggregating of spheres and plating on adhesive substrate, for N-CAM, vimentin, nestin, and A2B5, respectively. Bars =1.6 mm (A), 100 μm (B), 25 μm (C, E, F), 35 μm (D).

GCTM-2, which identifies undifferentiated ES cells[6], and did not express the early neuroectodermal marker N-CAM (data not shown). These distinct areas had a uniformly white–gray and opaque appearance under dark-field stereomicroscopy (Fig. 2A), and could be identified in 54% of the colonies (67/124). They were surrounded by cells with diverse morphologies expressing a large array of somatic and extraembryonic markers, including muscle actin and desmin[6], α-fetoprotein, hepatocyte nuclear factor (HNF)-α, cardiac actin, and kallikrein (RT-PCR; not shown).

Assuming that the cells in the distinct areas gave rise to the adjacent N-CAM$^+$ cells, clumps of about 150 cells were mechanically isolated from these areas and replated in serum-free medium[17]. Under these culture conditions, the clumps formed free-floating spherical structures within 24 h.

Supplementing the medium with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), a growth factor combination that is known to be effective for the propagation of human fetal- and adult-derived neuroepithelial progenitors[17–20], facilitated sequential propagation and expansion of the sphere cultures. During the first two weeks in culture, some cell death was observed and the spheres gradually acquired a uniform round morphology (Fig. 2B). A detailed analysis of marker expression and the growth and differentiation potential of the cells within the spheres was conducted in three preparations that were separately derived and propagated.

The level of proliferation of the cells within the spheres was monitored indirectly by measuring the increase in the volume of the spheres over time. Most of the cells within the spheres were viable as demonstrated by Trypan Blue staining (94 ± 3.2%, $n = 47$ spheres). A positive correlation between the volume of the spheres and the number of cells within the spheres (Fig. 3B) was documented at various passage levels (5–15 weeks after derivation), indicating that an increment in sphere volume could be used as an indirect indication of cell proliferation. The spheres grew over an 18- to 22-week period, after which time the volume of the spheres was stable or declined. A relatively rapid growth rate was observed during the first five to six weeks after derivation, with a population doubling time of ~4.7 days. It was followed by a 10- to 16-week period of slow and stable cell growth with a population doubling time of ~2.5 weeks. This proliferative capability could potentially allow a significant expansion of the progenitor cell cultures (Fig. 3A).

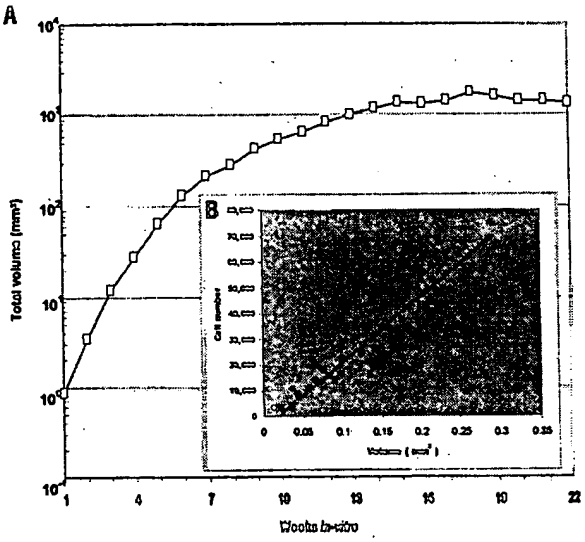

Figure 3. Cumulative growth curve for human ES-derived progenitor cells. (A) Continuous growth is evident during an 18- to 22-week period. The increment in the volume of the spheres was continuously monitored as an indirect measure of the increase in cell numbers. A linear positive correlation between the volume of the spheres and the number of cells within the spheres (B, insert) was maintained during cultivation.

Characterization of the progenitor cells within the spheres. Cells in the spheres expressed markers of neural progenitor cells, such as N-CAM (ref. 21; Fig. 2C), the intermediate-filament protein nestin[22] (immunostaining, Fig 2E; RT-PCR, Fig. 1A), A2B5 (ref. 23; Fig. 2F), vimentin[24] (Fig. 2D), and the transcription factor PAX-6 (Fig. 1A). The expression of these markers was maintained with prolonged cultivation *in vitro* (18 weeks).

To evaluate the proportion of neural progenitors in the cultures, spheres were disaggregated into single cells that were plated, fixed, and analyzed for the expression of the early neural markers (Fig. 2C–F). A high proportion of the cells expressed N-CAM (99 ± 1.6%, n = 11 experiments), nestin (97 ± 2.3%, n = 10 experiments), and A2B5 (90.5 ± 1.1%, n = 6). A lower proportion of cells were immunoreactive to the vimentin-specific antibody (67 ± 16.8%, n = 9 experiments). These proportions were stable during cultivation of the spheres (up to 18 weeks).

Oct-4 is a member of the POU-domain transcription factor family whose expression is limited in the mouse to pluripotent cells and is downregulated upon differentiation[25]. We have previously demonstrated a similar pattern of expression in human ES cells[6]. Oct-4 was not expressed by cells in the neural progenitor spheres, indicating that undifferentiated human ES cells were not present within the spheres (Fig. 1A).

To determine whether cells that had acquired markers of other tissues or lineages were present within the spheres, the expression of markers representing derivatives of mesoderm, endoderm, and epidermis were examined. Cells within the spheres expressed transcripts of markers of hematopoietic/endothelial progenitors (CD34, AC-133, Flk-1), endoderm (α1-antitrypsin, transferring, and amylase) and epidermis (keratin), as demonstrated by RT-PCR (Fig. 1B). Markers of extraembryonic endoderm were not expressed by the progenitors (α-fetoprotein and HNF-α, RT-PCR; not shown) or their differentiated progeny (low-molecular-weight cytokeratin and laminin immunostaining; not shown). The expression of transcripts of non-neural markers was evident after prolonged cultivation of the spheres. It could represent contamination by a small number of non-neural cells generated during the derivation of our cultures. Alternatively, it could represent plasticity of primitive neural progenitors that expressed markers, or gave rise to cells from other lineages[26,27]. Whatever the source, additional selection either on the basis of cell-surface markers[18] or on the expression of lineage-specific genes[12] may be needed to generate pure neural cultures.

In vitro neural differentiation. The neural progenitors in the spheres could differentiate *in vitro* into derivatives of the three fundamental neural lineages. In general, differentiation was induced by plating whole spheres on an appropriate substrate in the absence of growth factors. Under these conditions the spheres attached rapidly, and cells migrated out to form a monolayer of differentiated cells (Fig. 4A).

For neuronal differentiation studies, spheres were plated on poly-D-lysine and laminin-coated dishes. After two to three weeks, cells that migrated out and formed a monolayer both displayed the morphology and also expressed the structural markers that are characteristic of immature neurons, such as βIII-tubulin (Fig. 4B), the 70 kDa neurofilament proteins (Fig. 4C), and neuron-specific enolase (NSE; Fig. 1C). Moreover, the differentiated cells expressed markers of mature neurons such as the 160 kDa neurofilament proteins (NF-M, Fig. 4D; RT-PCR, Fig. 1C), MAP-2ab (Fig. 4E), and synaptophysin (Fig. 4F). Furthermore, the cultures contained cells that synthesized glutamate, expressed glutamic acid decarboxylase (GAD; the rate-limiting enzyme in GABA biosynthesis), synthesized GABA and serotonin, and expressed tyrosine hydroxylase (TH; Fig. 4G–K). Neurons that synthesized GABA and glutamate were relatively abundant, comprising 35% and 15% of the neuronal population, respectively. TH- and serotonin-producing cells were relatively rare (<1%).

For glial differentiation studies, spheres were plated on poly-D-lysine- and fibronectin-coated dishes and were cultured first in the presence of EGF, bFGF, and platelet-derived growth factor-AA (PDGF-AA), followed by culture in the presence of tri-iodothyronine (T3). The combination of bFGF and PDGF-AA is known to promote the proliferation of glial precursor cells[14], whereas T3 has been shown to enhance differentiation of oligodendrocyte lineage cells from human embryonic neural spheres[28].

Differentiation into astrocytes was demonstrated by the presence of cells that expressed glial fibrillary acidic protein (GFAP) (Fig. 4L; RT-PCR, Fig. 1C). Oligodendrocyte lineage cells were infrequent under our culture conditions and few cells were immunoreactive to O4, an antibody recognizing oligodendrocyte-specific glycolipids[29] (Fig. 4M). Differentiation to the oligodendrocyte lineage was further confirmed by demonstrating the expression of RNA transcripts of both myelin basic protein (MBP) and the *plp* gene (Fig. 1C). The *plp* gene encodes the proteolipid protein and its alternatively spliced product DM-20, which are major proteins of brain myelin[21].

To evaluate the proportion of neurons versus glial cells following induction of differentiation, spheres that were propagated 10 weeks were disaggregated and plated on poly-D-lysine- and laminin-coated dishes and cultured in the absence of mitogens for five days. Fifty-seven percent of the cells were immunoreactive to anti-βIII-tubulin (a marker characteristic of immature neurons) and 26% to anti-GFAP. Therefore, at least 83% of the cells took on a neural fate. The potential of the neural progenitors to give rise to both neurons and glial cells *in vitro* was maintained for the duration of the 22 weeks of propagation.

Integration and differentiation in host brain. To explore the developmental potential of the human ES-derived neural progenitors *in vivo*, disaggregated bromodeoxyuridine (BrdU)-labeled

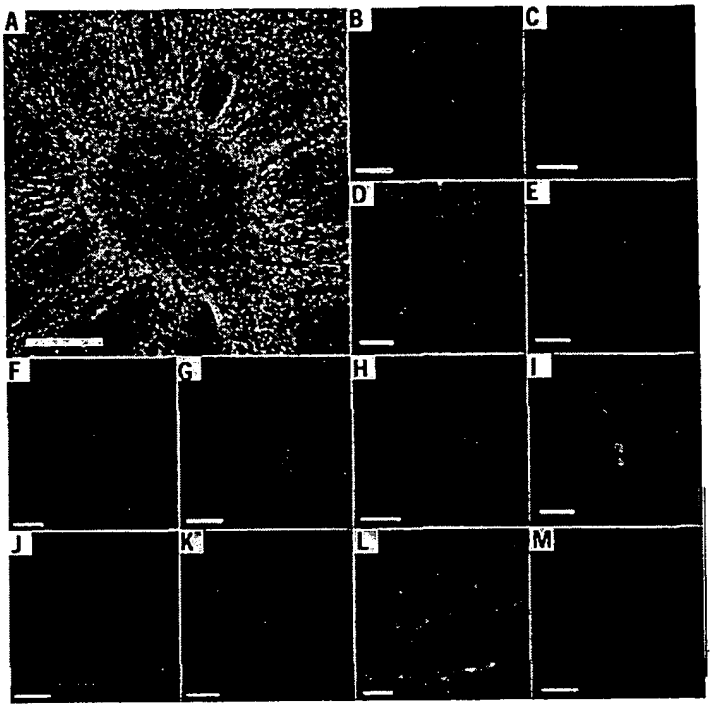

Figure 4. Phase contrast appearance and marker expression of differentiated cells originating from human ES-derived neural progenitor spheres. (A) Phase contrast micrograph of differentiated cells emanating from a sphere two weeks after plating onto an adhesive surface and culture in the absence of growth factors. (B–M) Indirect immunofluorescence microscopy of differentiated cells decorated with antibodies against the following neuronal and glial markers: β_{III}-tubulin (B), 70 kDa neurofilament proteins (C), 160 kDa neurofilament proteins (D), MAP2ab (E), synaptophysin (F), glutamic acid decarboxylase (G), GABA (H), glutamate (I), serotonin (J), tyrosine hydroxylase (K), GFAP (L), O4 (M). Bars = 200 μm (A, D), 50 μm (E), 20 μm (B, C, F–M).

spheres were implanted into the lateral cerebral ventricles of newborn mice[19]. Transplantation was performed 9–15 weeks after derivation of the neural spheres. Histological and immunochemical evaluation of serial brain sections was performed 4–6 weeks after transplantation. Numerous BrdU$^+$ cells were found in 9 out of 14 recipient animals, and successful engraftment was documented with donor cells from the three neural progenitor populations (Fig. 5). Transplantation efficiency was highly variable. BrdU$^+$ cells were not observed in the brain parenchyma of control animals that received transplantation of killed, BrdU-labeled, neural progenitors (Fig. 5D). The human origin of the cells decorated with anti-BrdU was confirmed by double labeling with a combination of anti-BrdU and anti-human specific ribonucleoprotein antibodies (Fig. 6A–D). The identity of the transplanted human cells was also confirmed by immunofluorescent staining with human-specific anti-mitochondrial antibodies (Fig. 6E–G). The distribution of cells that were immunoreactive with anti-BrdU and with human-specific anti-mitochondrial antibody in various regions of host brain was similar.

Brains that were examined a week after transplantation exhibited clusters of donor cells lining the ventricular wall (Fig. 5A). Four to six weeks following transplantation, human cells had left the ventricles and migrated in large numbers mainly as individual cells into the host brain parenchyma. The human cells demonstrated a widespread distribution in various regions of the host brain including periventricular areas, the entire length of the corpus callosum, fimbria, internal capsule, diencephalic tissue around the third ventricle, and dentate gyrus (Fig. 5B, E–H). Transplanted human cells also migrated anteriorily from the subventricular zone along the rostral migratory stream (Fig. 5C) and populated the olfactory bulb, indicating their potential to respond to local cues and migrate along established host brain tracts.

Differentiation *in vivo* into the three fundamental neural lineages was demonstrated by immunochemical studies using anti-human cell type–specific antibodies or double-labeling experiments with both anti-BrdU or anti-human specific ribonuclear protein (RNP) and anti-neural cell type–specific antibodies. Glial differentiation of the transplanted cells was abundant in the periventricular areas that consist of white-matter tracks where glial differentiation in the postnatal period is predominant. *In vivo* differentiation into astrocytes was demonstrated by immunochemical staining with anti-human-

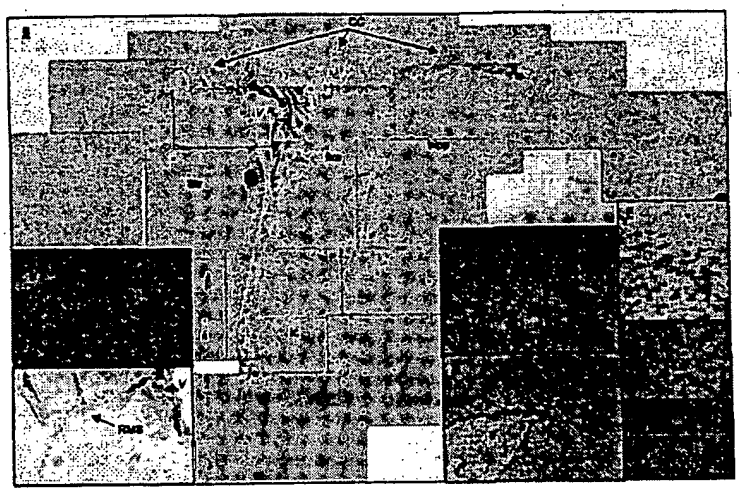

Figure 5. Dissemination of transplanted BrdU$^+$ human ES-derived neural progenitor cells in the mouse host brain. (A) Two days after transplantation, most cells were found lining the ventricular wall. (B) After four to six weeks, most cells had left the ventricles (V) and populated the corpus callosum (CC), fimbria (fim), and internal capsule (ic). (C) Chains of BrdU$^+$ cells were found in the RMS. (D) In animals that were transplanted with dead BrdU-labeled cells, there was no BrdU staining in the brain parenchyma. (E) BrdU$^+$ cells in the periventricular white matter. (F) High magnification of BrdU$^+$ cells in the corpus callosum; in the cortical layer above the corpus callosum (*) there were no BrdU$^+$ cells. (G) High-magnification image showing BrdU$^+$ cells populating the fimbria. (H) Low-magnification image showing BrdU$^+$ cells in the dentate gyrus. Str, striatum; hipp, hippocampus.

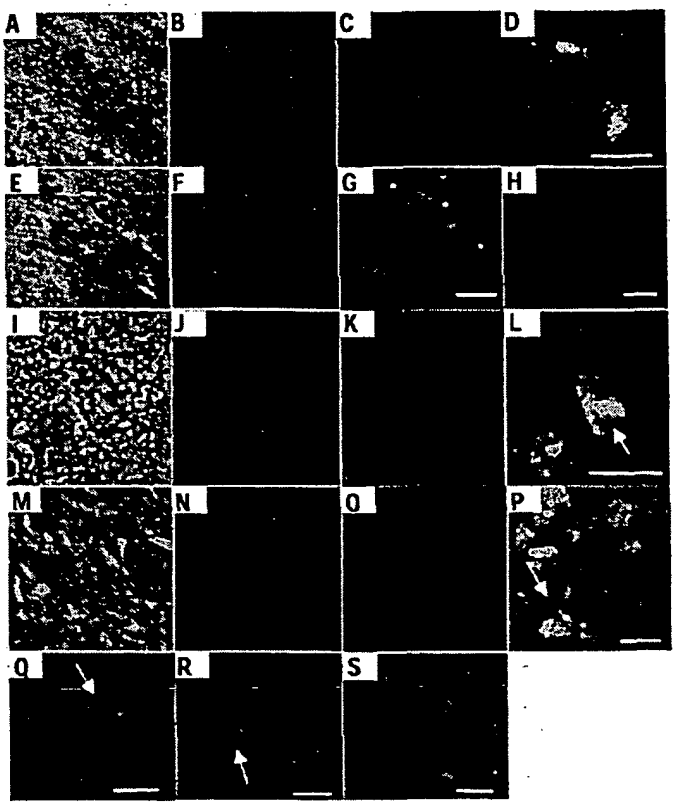

Figure 6. Identification of the transplanted cells in the brain by human and neural lineage–specific markers. (A–D) Nuclei (identified in Nomarski optics, panel A) were double labeled for BrdU (green fluorescence, panel B) and human-specific anti-RNP (red fluorescence, panel C). The nuclear co-localization (D) indicated that BrdU$^+$ cells were indeed of human origin. (E–G) A typical chain of transplanted cells in the corpus callosum, stained with human-specific anti-mitochondrial antibody. The mitochondrial staining (green fluorescence, panel F) on Nomarsky background (blue; panel G, cell nuclei indicated by asterisks) shows typical perinuclear localization. (H) A periventricular transplant-derived astrocyte detected by a human-specific anti-GFAP antibody. (I–L) A transplant-derived astrocyte from the periventricular region. The nucleus (identified by Nomarski optics, panel I) is labeled with BrdU (J, green fluorescence), indicating its origin from the graft and surrounded by GFAP staining (K, L). (M–P) A human oligodendrocyte progenitor cell identified in the periventricular region. The cell membrane (M, arrows) and nucleus (M, arrow and asterisk) are identified by Nomarski optics. Co-labeling of nucleus by anti-BrdU (N) and cell membrane by anti-NG-2 (O) are demonstrated in image (P). (Q) A CNPase$^+$ oligodendrocyte (green) in the corpus callosum, co-labeled with human-specific RNP (red). (R) A β_{III}-tubulin$^+$ neuron (green fluorescence) in the olfactory bulb, co-labeled with human-specific RNP (red). (S) Neuronal processes in the fimbria, stained with a human-specific anti-70 kDa neurofilament. Bars = 10 μm.

specific GFAP (Fig 6H) and by double labeling for BrdU and GFAP (Fig. 6I–L). Transplanted cells that differentiated into the oligodendroglial lineage were demonstrated by double immunostaining with anti-BrdU and anti NG-2 (a marker of oligodendrocyte progenitors[30]) or anti-human RNP and anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase; Fig. 6M–P and 6Q, respectively).

Neuronal differentiation of the human transplanted cells was specifically demonstrated in the olfactory bulb, a region where neuronogenesis occurs after birth (Fig. 6R). Neuronal processes of transplanted cells were also detected by a human-specific anti–light chain neurofilament antibody in the fimbria (Fig. 6S).

There was no histological evidence of teratoma or non-neural tissue formation in any of the recipient animals.

Discussion

Our findings show that a highly enriched population of proliferating neural progenitors may be derived from human ES cells. These neural progenitors are capable of extensive proliferation *in vitro* while retaining their potential to give rise to the three fundamental neural lineages and to participate in mammalian brain development. Derivation of proliferating, highly enriched tissue-specific progenitors from human ES cells, as exemplified here for the neural lineage, is expected to be highly valuable for the analysis of the stages of early development and for the development of a donor source for tissue reconstruction.

Our culture conditions promoted the undifferentiated proliferation of the human ES-derived neural progenitors for 20 weeks. Throughout cell propagation, expression of markers of early neuroectoderm was maintained, as was the progenitors' potential to differentiate into neurons and glial cells. The neural progenitors did not express markers of ES cells or the morphology and markers of differentiated neural cells. It should be noted that because the neural progenitors were not subjected to clonal analysis, it is not possible to determine whether our cultures contained multipotent human neural stem cells or a mixture of more restricted neural progenitors[31]. Nevertheless, our approach could distinguish among the early phases of human neurogenesis, including pluripotent ES stem cells, the proliferation of neural progenitors, and their differentiation into neurons and glial cells. This is the first step toward the development of more refined *in vitro* models that will distinguish between neural progenitors at various levels of commitment[26] and will allow the dissection of the cellular and molecular processes accompanying the various stages of development of the human nervous system.

Our data demonstrate that neural progenitors derived from human ES cells *in vitro* can respond appropriately to normal developmental cues *in vivo*. Following transplantation to the cerebral ventricles of newborn mice, the donor cells migrated in large numbers into the host brain parenchyma and became widely distributed. The engraftment efficiency was variable, and additional studies are required to determine to what extent, if at all, *in vivo* proliferation of transplanted cells contributed to the total number of human cells in host brains. Migration of the transplanted cells was not random, and the human progenitors followed established brain pathways, indicating that they respond to host's signals. The human ES cell–derived neural progenitors differentiated *in vivo* into neurons, astrocytes, and oligodendrocytes. Differentiation into neurons was demonstrated in the olfactory bulb where host differentiation into this lineage occurs in the postnatal period, whereas differentiation into astroglia and oligodendroglia was demonstrated in subcortical white-matter tracts where gliogenesis predominates and neurogenesis is complete[32]. These data demonstrate that cell fate was determined in a region-specific manner and according to the region's stage of development.

When ES cells, including those of human origin, are engrafted into various organs of young adult host mice, they may give rise to teratocarcinoma or teratomas[5,6]. We did not observe the formation of teratomas or non-neural tissues in any of the transplanted mice, and we also could not detect expression of the stem cell marker Oct-4 in the neural progenitor cultures. Thus, contamination by undifferentiated ES cells was probably eliminated by our selective derivation and propagation protocols. Nevertheless, given the expression of tran-

scripts of markers of non-neural lineages by the cells in our cultures, additional immunohistochemical and molecular studies are needed to determine whether non-neural human cells are generated in the host brain parenchyma. Thorough long-term studies are required to determine the safety of the transplantation of human ES cell–derived neural progeny, and to rule out potential hazards such as tumor formation or the development of cells from other lineages.

Data are accumulating rapidly regarding the signals and factors that govern the proliferation of neural progenitors and determine their fate during CNS development[31]. In this study we have used this knowledge to generate an enriched, expandable population of developmentally competent neural progenitors from human ES cells. This work serves as a platform for further manipulations with growth and differentiating factors that may eventually enable the derivation of specific neural cells[33], and may facilitate the use of human ES cells as a useful tool in basic neuroscience research and regenerative medicine.

Experimental protocol

Derivation and culture of progenitor cells. Human ES cells (HES-1 cell line[6]) with a stable normal (46XX) karyotype were cultured on mitomycin C mitotically inactivated mouse embryonic fibroblast feeder layer in gelatin-coated tissue culture dishes as described[6]. After three weeks of continuous culture, patches containing ~150 cells each were cut out from distinct areas within the differentiating ES colonies using the razor-sharp edge of a micro-glass pipette. Contamination by other cell types was avoided by paying careful attention to cut well within the distinct areas. The clusters of cells were transferred to plastic tissue culture dishes containing growth medium that consisted of Dulbecco's minimal essential medium (DMEM)/F12 (1:1), B27 supplementation (1:50), glutamine 2 mM, penicillin 50 units/ml, and streptomycin 50 μg/ml (Gibco, Gaithersburg, MD), and supplemented with 20 ng/ml human recombinant EGF and 20 ng/ml bFGF (R&D Systems, Inc., Minneapolis, MN). The clusters of cells developed into round spheres that were subcultured by dissection into quarters (by two no. 20 surgical blades; Swann-Morton, Sheffield, UK), every 7–21 days when their diameter exceeded 0.5 mm. Fifty percent of the medium was replaced every three to four days.

Analysis of growth. The increment in the volume of 24 spheres was monitored weekly starting from the first passage (one week after derivation). A stereomicroscope was used to measure the diameter of individual spheres, and their volume was calculated using the equation for the volume of a ball. The spheres were passaged every 7–21 days when the diameter of at least six spheres exceeded 0.5 mm. At each passage, six spheres (diameter >0.5 mm) were sectioned into quarters that were plated individually in a 24-well tissue culture dish. When growth was evaluated a week after passage, the sum of volumes of the daughter spheres was compared to the sum of volumes of the mother spheres.

Immunohistochemistry studies. Immunostaining of ES cell colonies to evaluate the expression of GCTM-2 and N-CAM was performed as described[6]. Standard protocols were used for the immunophenotyping of spheres, disaggregated progenitor cells, and differentiated cells. Fixation with 4% paraformaldehyde was used unless otherwise specified. Primary antibody localization was done by using swine anti-rabbit and goat anti-mouse immunoglobulins conjugated to fluorescein isothiocyanate (FITC 1:20; Dako, Carpinteria, CA), and goat anti-mouse IgM conjugated to Texas Red (1:50; Jackson Laboratories, West Grove, PA). Proper controls for primary and secondary antibodies revealed neither nonspecific staining nor antibody cross-reactivity.

To characterize the immunophenotype of cells within the aggregates, spheres that were cultivated 6–18 weeks were disaggregated and the single cells were plated on poly-D-lysine (30–70 kDa, 10 μg/ml; Sigma, St. Louis, MO) and laminin (4 μg/ml; Sigma), fixed after 4–12 h, and examined for the expression of N-CAM (acetone fixation, 1:10; Dako), nestin (rabbit antiserum, a gift of Dr. Ron McKay; 1:25), A2B5 (1:20; American Type Culture Collection, ATCC, Manassas, VA), and vimentin (methanol fixation, 1:20; Roche Diagnostics Australia, Castle Hill, NSW). Two hundred cells were scored within random fields (at 400×) for the expression of each of these markers, and the experiments were repeated at least three times.

For the study of the expression of extraembryonic endodermal markers, whole spheres were plated on poly-D-lysine and fibronectin (5 μg/ml; Sigma), cultured four weeks in growth medium without growth factors, and examined for the expression of low-molecular-weight (LMW) cytokeratin (Beckton Dickinson, San Jose, CA) and laminin (1:500; Sigma).

Neuronal differentiation was induced by culturing the spheres on poly-D-lysine and laminin in growth medium without supplementation of growth factors for two to three weeks. In some of the experiments, starting from the sixth day after plating, the medium was supplemented with all-*trans* retinoic acid (10^{-6} M; Sigma). Differentiated cells were analyzed for the expression of 160 kDa neurofilament protein (methanol fixation, 1:50; Chemicon, Temecula, CA), 70 kDa neurofilament protein (1:100; Chemicon), MAP2ab (1:100; Neomarkers, Union City, CA), glutamate (1:1,000; 1% (wt/vol) paraformaldehyde–1% (vol/vol) glutaraldehyde fixation; Sigma), synaptophysin (1:50; Dako), TH (Sigma), serotonin (1:1,000; Sigma), GAD (1:200, 1% (wt/vol) paraformaldehyde–1% (vol/vol) glutaraldehyde fixation; Chemicon; 1:200), GABA (1:1,000; Sigma), and β_{III}-tubulin (1:150; Sigma). To determine the proportion of neurons that synthesized the various neurotransmitters, at least 100 cells were scored within random fields of the outgrowth from differentiating spheres (at 400×) for the expression of β_{III}-tubulin and each of the neurotransmitters, and the experiments were repeated at least three times.

To enhance the differentiation toward the glial lineages, spheres were plated on poly-D-lysine and fibronectin, cultured two weeks in growth medium supplemented with recombinant human PDGF-AA (20 ng/ml), bFGF (20 ng/ml), and EGF (20 ng/ml), followed by two weeks in the presence of T3 (30 nM; Sigma) only. Differentiated cells were analyzed for the expression of GFAP (1:50; Dako) and O4 (1:10; Chemicon).

Reverse transcription (RT)-PCR analysis. Total RNA was collected from human ES cell colonies (one week after passage), from free-floating spheres, and from differentiated cells growing from spheres that were induced to differentiate to the neuronal or glial lineages as detailed above. Total RNA was isolated using RNA STAT-60 solution (TEL-TEST, Inc., Friendswood, TX) and was reverse-transcribed into complementary DNA (cDNA) with SuperScript First Strand Synthesis System (Gibco) using oligo (dT) as a primer according to the manufacturer's instructions. PCR was carried out using standard protocols with *Taq* DNA Polymerase (Gibco) or *Tfl* DNA Polymerase (Promega, Madison, WI). Primer sequences (forward and reverse) and the length of amplified products were as follows: Oct-4 (primers[34]); nestin, PAX-6, NSE, NF-M, *plp* (primers[35]); keratin, amylase, α1-antitrypsin (primers[8]); flk-1, CD34, AC133 (primers[36]); GFAP, MBP (primers[20]); *transferrin:* 5′-CTGACCTCACCTGGGACAAT-3′, 5′-CCATCAAGGCACAGC-3′ (367 bp); α-fetoprotein: 5′-CCATGTACATGAGCACTGTTG-3′, 5′-CTCCAATAACTCCTGGTATCC-3′ (338 bp); HNF-α: 5′-GAGTTTACAGGCTTGTGGCA-3′, 5′-GAGGGCAATTCCTGAGGATT-3′ (390 bp). As a control for messenger RNA (mRNA) quality, β-actin transcripts were assayed using the same RT-PCR and the following primers: 5′-TCACCACCACGGCCGAGCG-3′, 5′-TCTCCTTCTGCATCCTGTCG-3′ (291 bp). Amplification conditions were as follows: 94°C for 4 min followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, 72°C for 45 s, and extension at 72°C for 7 min. Products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

Transplantation to the developing brain. Spheres were cultured in the presence of BrdU (50 μM; Sigma) for 10 days. Fifty percent of the medium was replaced every three to four days with fresh medium containing fresh BrdU. The spheres were then disaggregated; 86% of the cells were viable as determined by Trypan Blue staining, and 40% were decorated by anti-BrdU. Approximately 50,000–100,000 cells (in 2 μl PBS) were injected into the lateral ventricles of newborn (P1) mice (Sabra mice; Harlan, Jerusalem, Israel) by using a glass micropipette (300 μm outer diameter) connected to a micro-injector (Narishigi Inc., Tokyo, Japan). Transplantation of dead, BrdU-labeled, human ES cell–derived neural progenitors served as control experiments. The neural progenitors underwent three cycles of freezing by plunging into liquid nitrogen and thawing in room temperature just before transplantation. At one to six weeks of age, recipients were anesthetized and perfused with 4% paraformaldehyde in PBS.

Detection and characterization of donor human neural progenitors *in vivo*. Serial 7-μm frozen sections were examined by immunostaining after postfixation with acetone or with 4% paraformaldehyde. The transplanted cells were detected by immunostaining with antibodies for BrdU

(1:20; Dako), anti-human specific RNP antibody (1:20; Chemicon), and anti-human specific mitochondrial antibody (1:20; Chemicon). BrdU antibody was detected by using the peroxidase-conjugated Vectastain kit (Vector Laboratories, Burlingame, CA), developed with diaminobenzidine (DAB), or by using goat anti-mouse IgG conjugated to Alexa 488 (1:100; Jackson). Anti-RNP and mitochondrial antibodies were detected with goat anti-mouse IgM conjugated to Cy5 and goat anti-mouse IgG conjugated to Alexa488, respectively (1:100; Jackson). Transplanted astrocytes were identified by double staining for BrdU and GFAP (1:100; Dako) or by anti-human specific GFAP (1:100; Sternberger Monoclonals Inc., Lutherville, MD). Anti-CNPase (1:100; Sigma) and anti NG2 (1:100; Chemicon) were used for the oligodendrocyte lineage. Neurons were detected by immunostaining with human-specific anti-neurofilament light chain (1:100; Chemicon) and anti-β_{III}-tubulin (antibody as detailed above; 1:100). Goat anti-rabbit conjugated to Cy5 (1:100; Jackson) and goat anti-mouse IgG conjugated to Alexa488 (1:100; Jackson) were used for detection of primary antibodies. Images were taken with a confocal microscope (Zeiss). All double-stain immunofluorescence signals were analyzed at multiple consecutive planes to ensure the co-localization of nuclear and cytoplasmic or membranal signals to the same cell.

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EXHIBIT I

STEM CELLS®
Original Article

Transplantation of Human Embryonic Stem Cell–Derived Neural Progenitors Improves Behavioral Deficit in Parkinsonian Rats

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Key Words. Parkinson's disease • Cell therapy • Human ES cells

ABSTRACT

Human embryonic stem cells (hESCs) may potentially serve as a renewable source of cells for transplantation. In Parkinson's disease, hESC-derived dopaminergic (DA) neurons may replace the degenerated neurons in the brain. Here, we generated highly enriched cultures of neural progenitors from hESCs and grafted the progenitors into the striatum of Parkinsonian rats. The grafts survived for at least 12 weeks, the transplanted cells stopped proliferating, and teratomas were not observed. The grafted cells differentiated in vivo into DA neurons, though at a low prevalence similar to that observed following spontaneous differentiation in vitro. Transplanted rats exhibited a significant partial correction of D-amphetamine and apomorphine-induced rotational behavior, along with a significant improvement in stepping and placing nonpharmacological behavioral tests. While transplantation of uncommitted hESC-derived neural progenitors induced partial behavioral recovery, our data indicate that the host-lesioned striatum could not direct the transplanted neural progenitors to acquire a dopaminergic fate. Hence, induction of their differentiation toward a midbrain fate prior to transplantation is probably required for complete correction of behavioral deficit. Our observations encourage further developments for the potential use of hESCs in the treatment of Parkinson's disease. *Stem Cells 2004;22:1246–1255*

INTRODUCTION

Pharmacological treatments of Parkinson's disease have limited long-term success and are associated with serious motor side effects. Transplantation of dopaminergic (DA) neurons is an alternative potential therapeutic approach, and clinical relief of Parkinsonism has been demonstrated in some patients following implantation of fetal-derived DA neurons [1–3]. However, limited donor tissue supply, ethical considerations, and complicating dyskinesias are major limitations of this mode of therapy.

Pluripotent human embryonic stem cells (hESCs) [4, 5], which potentially proliferate indefinitely in culture, may supply unlimited numbers of DA neurons for transplantation. The potential of mouse ES cells to generate functional

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DA neurons and to correct behavioral deficits after engraftment into Parkinsonian rats has been demonstrated [6, 7]. When low numbers of undifferentiated mouse ES cells were implanted into the rat DA-depleted striatum, the cells proliferated and differentiated into functional DA neurons that reduced Parkinsonism. However, lethal teratomas developed in 20% of the animals [6]. In an alternative approach, highly enriched populations of midbrain neural precursors were developed in vitro from mouse ES cells and then implanted into Parkinsonian rats. The engrafted cells led to the recovery from Parkinsonism, and teratoma tumor formation was not observed [7]. The potential therapeutic effect of engrafted hESCs in a Parkinsonian animal model has not yet been evaluated.

We previously derived highly enriched cultures of neural progenitors (NPs) from hESCs [8]. These NPs may serve as a platform for generating DA neurons to treat Parkinsonism. Here, we have implanted the human NPs into Parkinsonian rats. We demonstrate the long-term survival of the graft, lack of teratoma formation, spontaneous differentiation of a relatively small fraction of the transplanted cells into DA neurons, and a significant partial behavioral improvement in the transplanted animals.

Materials and Methods

Cell Culture

hESCs (HES-1 cell line) [5] with a stable normal (46XX) karyotype were cultured on a mitomycin C–treated mouse embryonic fibroblast feeder layer in gelatin-coated tissue culture dishes, as previously described [5]. To induce neural differentiation, clumps of undifferentiated hESCs were plated on fresh mitotically inactivated feeders and cultured for 8 days in serum-containing medium comprised of Dulbecco's Modified Eagle's Media (DMEM; Gibco, Gaithersburg, MD) containing glucose at 4500 mg/L without sodium pyruvate, supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, 2 mM glutamine, penicillin at 50 U/ml, streptomycin (Gibco) at 50 μg/ml, and noggin (R&D Systems Inc., Minneapolis) at 500 ng/ml. The medium was replaced every other day. Noggin was then omitted, and the cells were further cultured in the same medium for an additional 6 days. At this time, 70%–90% of the colonies differentiated almost uniformly into tightly packed small cells with a uniform gray opaque appearance under dark field stereomicroscopy. Patches containing about 150 cells each were cut out from the gray opaque areas using a razor blade (surgical blade #15), then replated in serum-free medium that consisted of DMEM/F12 (1:1), B27 supplementation (1:50), 2 mM glutamine, penicillin at 50 U/ml, and streptomycin (Gibco) at 50 μg/ml, supplemented with 20 ng of human recombinant epidermal growth factor per ml, and 20 ng of basic fibroblast growth factor per ml (R&D Systems, Inc.). The clusters of cells developed into round spheres that were subcultured once a week, as previously described [8]. The medium was replaced twice a week.

Immunocytochemical Studies

Differentiation was induced on laminin as previously described [8]. Cells were fixed with 4% paraformaldehyde, either 18 hours (for NP markers) or 1 week (for neuronal markers) after plating, then stained for neural cell adhesion molecule (NCAM) (1:10; Dako, Carpinteria, CA), nestin (1:200), polysialic acid NCAM (PSA-NCAM) (1:200; both from Chemicon, Temecula, CA), A2B5 (1:20; American Type Culture Collection [ATCC], Manassas, VA), β-tubulin III (1:2000) and serotonin (1:1000; both from Sigma, St. Louis), and tyrosine hydroxylase (TH) (1:100; Pel-Freez, Rogeus, AZ). Primary antibody localization was performed by using swine anti-rabbit and goat anti-mouse immunoglobulins conjugated to fluorescein isothiocyanate (FITC) (Dako, A/S Denmark; 1:20–50), goat anti-mouse immunoglobulin M (IgM) conjugated to FITC (Jackson Laboratory, West Grove, PA; 1:100), goat anti-rabbit Ig conjugated to Texas Red (Jackson Laboratory; 1:100), and goat anti-mouse IgG conjugated to $Cy^{TM}3$ (Jackson Laboratory; 1:500). Proper controls for primary and secondary antibodies revealed neither nonspecific staining nor antibody cross-reactivity. To determine the percentage of specific cell types, 200–500 cells were scored within random fields for each marker in three separate experiments.

RT-PCR Analysis

Total RNA was extracted from (a) hESC colonies (1 week after passage), (b) free-floating spheres after 6 weeks in culture, and (c) differentiated cells growing from the spheres 1 week after plating in differentiation-inducing conditions, as detailed above. The medium that was used to induce differentiation was supplemented with the combination of 400 μM ascorbic acid (Sigma) and the survival factors NT3 at 10 ng/ml, NT4 at 20 ng/ml, and BDNF at 10 ng/ml (all human recombinants from R&D Systems Inc.). Total RNA was isolated using RNA STAT-60 solution (TEL-TEST, Inc., Friendswood, TX) or TRI-reagent (Sigma), followed by treatment with RNase-free DNase (Ambion, RNA Company, Austin, TX). The cDNA synthesis was carried out using Moloney murine leukemia virus reverse transcriptase (RT) and oligo as a primer, according to the manufacturer's instructions (Promega, Madison, WI). To analyze relative expression of different mRNAs, the amount of cDNA was normalized based on the signal from glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) mRNA. Levels of different mRNAs expressed by neural spheres and differentiated cells were compared with that in the undifferentiated hESCs. Polymerase chain reaction (PCR) was carried out using standard protocols with Taq DNA polymerase (Gibco). Amplification conditions were as follows: denaturation at 94°C for 15 seconds, annealing at 55–60°C for 30 seconds, and extension at 72°C for 45 seconds. The number of cycles varied between 18 and 40, depending on the abundance of particular mRNA. Primer sequences (forward and reverse 5'-3') and the length of the amplified products were as follows:

Oct4 (CGTTCTCTTTGGAAAGGTGTTC, ACACTCGGACCACGTCTTTC; 320 bp)
Otx2 (CGCCTTACGCAGTCAATGGG, CGGGAAGCTGGTGATGCATAG; 641 bp)
Pax2 (TTTGTGAACGGCCGGCCCCTA, CATTGTCACAGATGCCCTCGG; 300 bp)
Pax5 (CCGAGCAGACCACAGAGTATTCA, CAGTGACGGTCATAGGCAGTGG; 403 bp)
En1 (CTGGGTGTACTGCACACGTTAT, TACTCGCTCTCGTCTTTGTCCT; 357 bp)
En2 (GTGGGTCTACTGTACGCGCT, CCTACTCGCTGTCCGACTTG; 368 bp)
Lmx1B (TCCTGATGCGAGTCAACGAGTC, CTGCCAGTGTCTCTCGGACCTT; 561 bp)
Nurr1 (GCACTTCGGCAGAGTTGAATGA, GGTGGCTGTGTTGCTGGTAGTT; 491 bp)
Ptx3 (TGGGAGTCTGCCTGTTGCAG, CAGCGAACCGTCCTCTGGG; 372 bp)
TH (GTCCCCTGGTTCCCAAGAAAAGT, TCCAGCTGGGGGATATTGTCTTC; 331 bp)
AADC (CTCGGACCAAAGTGATCCAT, GGGTGGCAACCATAAAGAAA; 252 bp)
Pax6 (AACAGACACAGCCCTCACAAACA, CGGGAACTTGAACTGGAACTGAC; 274 bp)
Nkx2.2 (ACGAATTGACCAAGTGAAGCTAC, AACCCGGGCTGCGGCTGCAGGAAT; 379 bp)
Olig2 (GCTGTGGAAACAGTTTGGGT, AAGGGTGTTACACGGCAGAC; 291 bp)
HB9 (CAAGAAACAGCGAGAGGGAG, AACGCTCGTGACATAATCCC; 300 bp)
β-actin (CGCACCACTGGCATTGTCAT, TTCTCCTTGATGTCACGCAC; 200 bp)
GAPDH (AGCCACATCGCTCAGACACC, GTACTCAGCGCCAGCATCG; 301 bp)

Sphere Transplantation

6-Hydroxydopamine (8 μg/rat) was stereotaxically injected in 4 μl into the right substantia nigra of male Sprague-Dawley rats (weighing 250–280 g; coordinates of injection: P = 4.8, L = 1.7, H = –8.6 from bregma). Eighteen days after 6-hydroxydopamine injection, rats were selected for transplantation if they had >350 rotations per hour after s.c. injection of apomorphine (25 mg/100 g body weight) and, if 2 days later, they also had >360 (mean 520 ± 38) rotations per hour after i.p. injection of D-amphetamine (4 mg/kg). Two to five days later, partially mechanically dissociated spheres (passaged for 6 weeks) were transplanted (400,000 cells in 12–14 μl/animal [7]) along two tracts per striatum [6] (coordinates: anteromedial tract, A = 1, L = 2, H = –7.5 to –4; posterolateral tract, A–P = 0, L = 3.5, H = –7.5 to –4.5). Control sham-operated rats were injected with vehicle solution. All rats received daily i.p. injections of 10 mg cyclosporine A per kg (Sandimmune; Novartis, Basel, Switzerland).

Behavioral Tests

At 2 weeks and 1, 2, and 3 months after transplantation, the severity of the disease was scored by pharmacological tests in sphere- and vehicle-transplanted animals. Rotations were counted for 1 hour after s.c. injection of apomorphine, and again 2 days later after i.p. D-amphetamine by a computerized rotometer system (San-Diego Instruments, San Diego).

Nonpharmacological tests were performed at 2 weeks and 3 months after transplantation. These included stepping adjustments [9] and forelimb placing [10] tests. The number of stepping adjustments was counted for each forelimb during slow sideway movements in forehand and backhand directions over a standard flat surface. The stepping adjustments test was repeated three times for each forelimb during three consecutive days. The forelimb-placing test assesses the rats' ability to make directed forelimb movements in response to a sensory stimulus. Rats were held with their limbs hanging unsupported. They were then raised to the side of a table so that their whiskers made contact with the top surface while the length of their body paralleled the edge of the tabletop. Control rats place their forelimb on the tabletop in response to whisker stimulation almost every time, whereas injured rats do not. Each test included 10 trials of placement of each forelimb and was repeated daily for three consecutive days. The results of both tests are expressed as percentage of forelimb stepping adjustments and placing in the lesioned side, as compared with the nonlesioned side. All behavioral tests were performed blinded to treatment.

Brain Immunohistochemistry

Rats were euthanized by pentobarbital overdose and perfused with saline and 4% paraformaldehyde. Serial 8 μm coronal frozen sections were prepared, and every seventh section was stained with hematoxylin and eosin (H&E) to identify and map the grafts' location. Immunofluorescent stainings were performed following 4% formaldehyde fixa-

tion with the following primary antibodies: human-specific mitochondria (MITO, 1:20); TH (1:100), nestin (1:50), Musashi (1:400), neuronal nuclei marker (NeuN, 1:50), human dopamine transporter (DAT, 1:2000), human-specific ribonuclear protein (RNP, acetone fixation, 1:20), proliferating cell nuclear antigen (PCNA, methanol fixation, 1:100; all from Chemicon); glial fibrillary acidic protein (GFAP) (1:200), NCAM (1:10), CD44 (1:20; all from Dako); neurofilament heavy chain (NF, 1:100), β-tubulin III (1:50; both from Sigma); and Ki67 antigen (1:100; Novocastra Laboratories, Newcastle, U.K.). Goat anti-mouse IgG conjugated to Alexa 488 or Cy3, goat anti-mouse IgM conjugated to Texas Red, goat anti-rabbit IgG conjugated to Alexa 488 or to Texas Red (Jackson Laboratory; 1:100), and goat anti-rat IgG conjugated to Alexa 488 (Molecular Probes, Eugene, OR; 1:500) were used where appropriate for detection of primary antibodies. The number of 4',6'-diamidino-2-phenylindole hydrochloride (DAPI)$^+$ nuclei within the grafts was counted every fourth section in eight animals using a computerized image analysis system (Image-Pro version 4.1, Media Cybernetics, Silver Spring, MD). The graft borders were defined by anti-human-specific mitochondria staining. Cell counts from serial sections were corrected according to the Abercrombie method (1946) and adjusted to the total size of the graft [11]. From the 10 animals that were evaluated for amphetamine-induced rotational behavior, serial brain sections were available in six animals for counting of TH$^+$ neurons. TH$^+$ neurons were counted every fourth section, double-labeled for TH and human mitochondria, and adjusted for the whole graft following Abercrombie correction (1946) [11]. Images were taken by a fluorescent (Nikon E600, Kanagawa, Japan) and a confocal microscope (Zeiss, Feldbach, Switzerland) to ensure colocalization of nuclear, cytoplasmic, or membranal signals to the same cell.

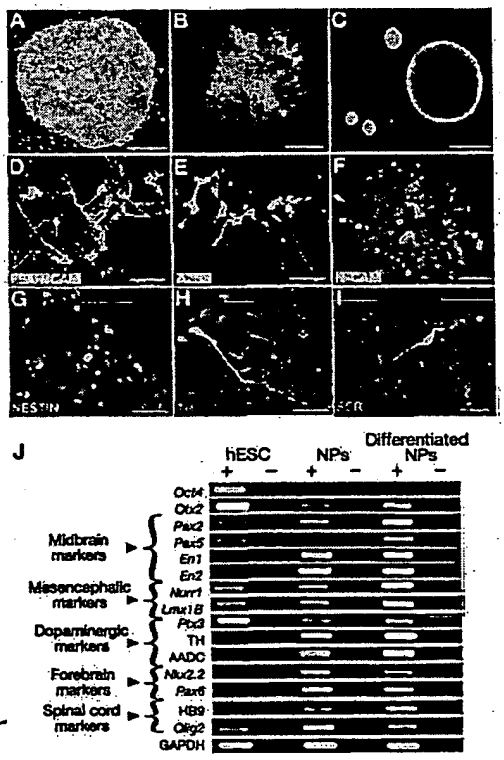

Figure 1. Derivation and characterization of spheres. Dark field stereomicroscope images of (A) an undifferentiated hESC colony 1 week after passage; (B) noggin-treated colony at 2 weeks after passage; and (C) hESC-derived spheres. Immunostaining of the progenitors for (D) PSA-NCAM, (E) A2B5, (F) NCAM, and (G) nestin. Double immunolabeling showing differentiated NPs co-expressing β-tubulin III and (H) TH or (I) serotonin, respectively. Semiquantitative reverse transcription–polymerase chain reaction demonstrating the expression of (J) *Oct4*, markers along dopaminergic neuron development, forebrain and spinal cord markers within cultures of hESCs, and undifferentiated and differentiated NPs. A ± indicates presence or absence of reverse transcriptase. Space bars: (A, B), 100 μm; (C), 200 μm; (D–I), 50 μm. Abbreviations: hESC, human embryonic stem cell; NP, neural progenitor; PSA-NCAM, polysialic acid neural cell adhesion molecule; TH, tyrosine hydroxylase.

Statistical Analysis

Values in the behavioral tests and cell counts are given as mean ± standard error. The mean number of rotations in the pharmacological behavioral tests, at baseline and after 12 weeks, were compared by Student's *t*-test. The mean results (in percentage) of nonpharmacological tests were also compared among the experimental groups using Student's *t*-test. One-tailed analysis of variance, followed by the Bonferroni post hoc test, was used for multiple comparisons in the pharmacological behavioral tests. Pearson's correlation coefficient was calculated to determine the association between total number of TH$^+$ neurons and recovery of amphetamine-induced rotational behavior. A $p < .05$ was considered statistically significant.

Results

Development and Characterization of hESC-Derived NPs

Differentiation of hESCs into highly enriched cultures of NPs was accomplished according to our two-step protocol [8] with some modifications [12]. In the first step, hESC colonies (Fig. 1A) were cultured for prolonged periods on feeders in the presence of the bone morphogenetic protein antagonist noggin. Under these culture conditions, in most colonies, the hESCs differentiated almost uniformly into tightly packed small progenitors. In parallel, the colo-

nies acquired a nearly uniform gray opaque appearance under dark field stereomicroscope (Fig. 1B). A detailed characterization of these progenitors and the effect of noggin on the differentiation of hESCs are reported elsewhere [12]. In the second step, clumps of 150 progenitors were isolated from the colonies and further propagated for 6 weeks as spheres (Fig. 1C) in serum-free medium supplemented with mitogens [8]. Prior to transplantation, the phenotype of the cells within the spheres and their potential to develop into midbrain DA neurons was characterized. Indirect immunofluorescence analysis (Figs. 1D–G) demonstrated that >90% of the cells within the spheres expressed NP markers (PSA-NCAM 94.7% ± 2.4%, A2B5 90.7% ± 3.2%, NCAM 91.3% ± 2.2%, nestin 94.7% ± 0.7%). Thus, the spheres were highly enriched for NPs.

Successful differentiation of the hESC-derived NPs into midbrain DA neurons probably requires the induction of the same key regulatory genes that are expressed by NPs during the development of the midbrain in vivo. These genes include the *Otx*, *Pax2*, *Pax5*, *En1*, *En2*, *Nurr1*, and *Lmx1b* genes [13]. Markers of DA neurons and regulatory genes in their development were expressed by the NPs, suggesting that they had the developmental potential to differentiate into midbrain DA neurons. Markers of other brain areas were also expressed by the spheres. Oct4, a marker of undifferentiated hESCs, was not detected, suggesting that the spheres did not include undifferentiated cells (Fig. 1J).

We further characterized the phenotype of the NPs following spontaneous differentiation in vitro. Upon withdrawal of mitogens from the medium and plating on laminin, the spheres attached rapidly, and cells migrated out to form a monolayer of differentiated cells. After 7 days of differentiation, the expression of the regulatory genes of midbrain development and markers of DA neurons was upregulated in the differentiated progeny (Fig. 1J). Double-labeling studies showed that 29.0% ± 0.6% of the cells were immunoreactive with anti-β-tubulin III (a neuronal marker), 0.56% ± 0.05% of the cells co-expressed β-tubulin III and TH (Fig. 1H), and 0.89% ± 0.11% co-expressed β-tubulin III and serotonin (Fig.1I). These results suggested that a low percentage (<1%) of the progenitors spontaneously differentiated into putative midbrain or hindbrain neurons.

Survival and Differentiation after Transplantation to Parkinsonian Rats

We next explored the survival, differentiation, and function of the NPs after transplantation into the right striatum of Parkinsonian rats. First, 6-hydroxydopamine was injected into the right substantia nigra to deplete dopaminergic innervation in the ipsilateral striatum. At 3 weeks after formation of the lesion, hESC-derived neural spheres that had been passaged for 6 weeks were grafted into the right striatum of rats that were preselected for pharmacological-induced high rotational activity. The rats were sacrificed for histopathological analysis of the graft 24 hours after transplantation ($n = 6$ animals), and after behavioral follow-up of 12 weeks (21 sphere- and 17 vehicle-grafted rats).

TH immunohistochemistry showed a rich fiber network in the striatum contralateral to the lesion (Fig. 2A), but there was no staining on the lesioned side (Fig. 2B). The grafts were easily identified in brain sections following H&E staining (Fig. 2C), fluorescent DAPI nuclear counterstaining, and indirect immunofluorescence staining for human-specific markers. At 12 weeks after transplantation, a graft was not identified in two rats, and the brains of three rats were processed for RT-PCR analysis (see below). In each of the remaining 16 animals, two grafts were found, most often as a tubular mass of cells along the needle tract within the striatum. In five animals, one of the two grafts was ectopic and was observed as a round mass in the cortex. An inflammatory process was not observed in the transplanted striata or in the control-depleted striata.

We used human-specific anti-mitochondria (Fig. 2D) and anti-RNP (Fig. 2E) antibodies to specifically identify human cells in transplanted rat brain sections. At 12 weeks post-transplantation, cells that were immunoreactive with the anti-human mitochondria antibodies were found only along the needle tract and within the striatum, and there was no indication of cell migration to neighboring brain regions.

The number of cells within the grafts was evaluated by counting the number of nuclei within the human mitochondria positively stained grafts in serial sections. At 12 weeks post-transplantation, the average number of human cells per striatum was 294,774 ± 72,401 ($n = 8$), 73.5% of the initial 400,000 cells that were injected into each animal. Survival of the transplanted cells, as well as their proliferation (see below), could contribute to this figure.

Immunostaining with human-specific and neural markers showed cells within the grafts that maintained the phenotype of undifferentiated NPs and expressed nestin, mushashi-1, and NCAM (Fig. 2F–H), as well as differentiated cells expressing the astroglial progenitor marker CD44, the astrocyte marker GFAP, and the neuronal markers heavy chain NF and NeuN (Fig. 2I–L). Double staining for human mitochondria and TH showed the presence of graft-derived TH^+ cells and fibers (Fig. 2M–Q). TH^+ fibers were commonly observed within the area of distribution of the grafted cells in the host striatum, with some outgrowth from this area into the host striatum (Fig. 2M). In vehicle-grafted animals, there were no TH^+ cells in the ipsilateral striatum.

At 12 weeks post-transplantation, the number of TH^+ cells in the human mitochondria–stained areas was counted

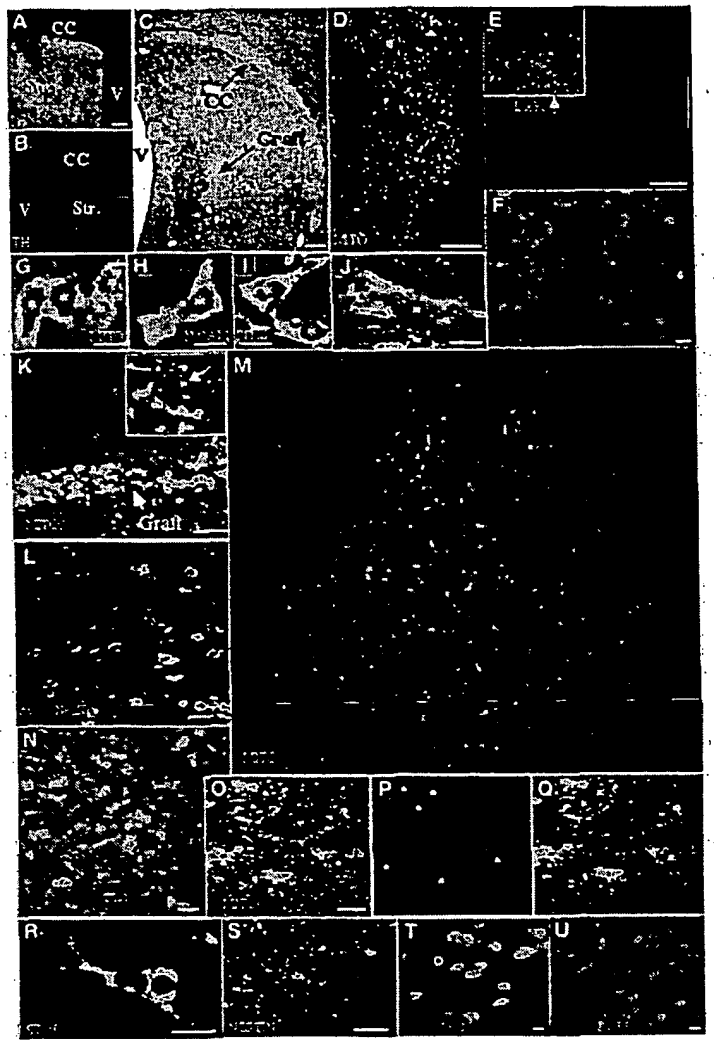

Figure 2. Immunohistochemical characterization of transplanted neural progenitors. Immunofluorescence staining for TH in the (A) nonlesioned and (B) lesioned striatum 18 days after 6-hydroxy-dopamine injection. (C): Hematoxylin and eosin staining of a graft 12 weeks after transplantation. (D): Low power field showing the elongated vertical graft in the striatum decorated with human-specific MITO, surrounded by rat tissue. (E): Human identity of cells was confirmed by staining with an anti-RNP antibody (inset: DAPI nuclear counterstain). (F): Immunoreactivity of cells within the graft with human-specific antinestin. (G–J): Confocal microscopy of immunofluorescent-stained sections at 12 weeks post-transplantation showed some human cells expressing markers of neural precursors and also cells expressing glial markers. Confocal images showing grafted cells expressing (G) human mitochondria and mushashi-1, (H) human-specific N-CAM, (I) human-specific CD44, and (J) human mitochondria and GFAP (nuclei indicated by *). (K): Low power image of brain section double stained with antihuman mitochondria and anti–heavy chain NF with DAPI nuclear counterstain, showing that the majority of the transplant did not stain with the neuronal marker. Some NF$^+$ human cells (inset, arrow) were identified mainly near the interface of the graft. (L): Nuclear coexpression of human RNP and NeuN. (M): Double immunostaining of the graft in the striatum showing TH$^+$ cells and fibers within the human mitochondrial–stained graft, with some outgrowth of TH$^+$ fibers at the periphery of graft, into the human mitochondria–negative rat tissue. (N–R): Generation of dopaminergic cells from the transplants. (N): A cell coexpressing TH and human mitochondria within the graft. Confocal microscopy images confirmed the coexpression of human mitochondria and TH [(O): merged image; (P): TH alone; (Q): human mitochondria alone, Nomarsky optics background] or human mitochondria and DAT (R) within the same cells. The nuclei (located by Nomarsky optics) are indicated by asterisks. (S): Immunostaining of the graft for nestin at 24 hours after transplantation. (T–U): Transplanted cells stop proliferating after transplantation. Multiple PCNA$^+$ cells were found at (T) 24 hours post-transplantation but not at (U) 12 weeks after transplantation. Space bars: (G–J) and (L–U), 10 μm; (A, B, D–F, K), 50 μm; (C), 200 μm. All panels apart from (A, B, S, and T) show stainings of grafts 12 weeks after transplantation. Abbreviations: CC, corpus callosum; DAPI, 4',6'-diamidino-2-phenylindole hydrochloride; DAT, human dopamine transporter; GFAP, glial fibrillary acidic protein; MITO, antimitochondria; N-CAM, neural cell adhesion molecule; NeuN, neuronal nuclei marker; NF, neurofilament; PCNA, proliferating cell nuclear antigen; RNP, ribonuclear protein; Str, striatum; TH, tyrosine hydroxylase; V, brain ventricle.

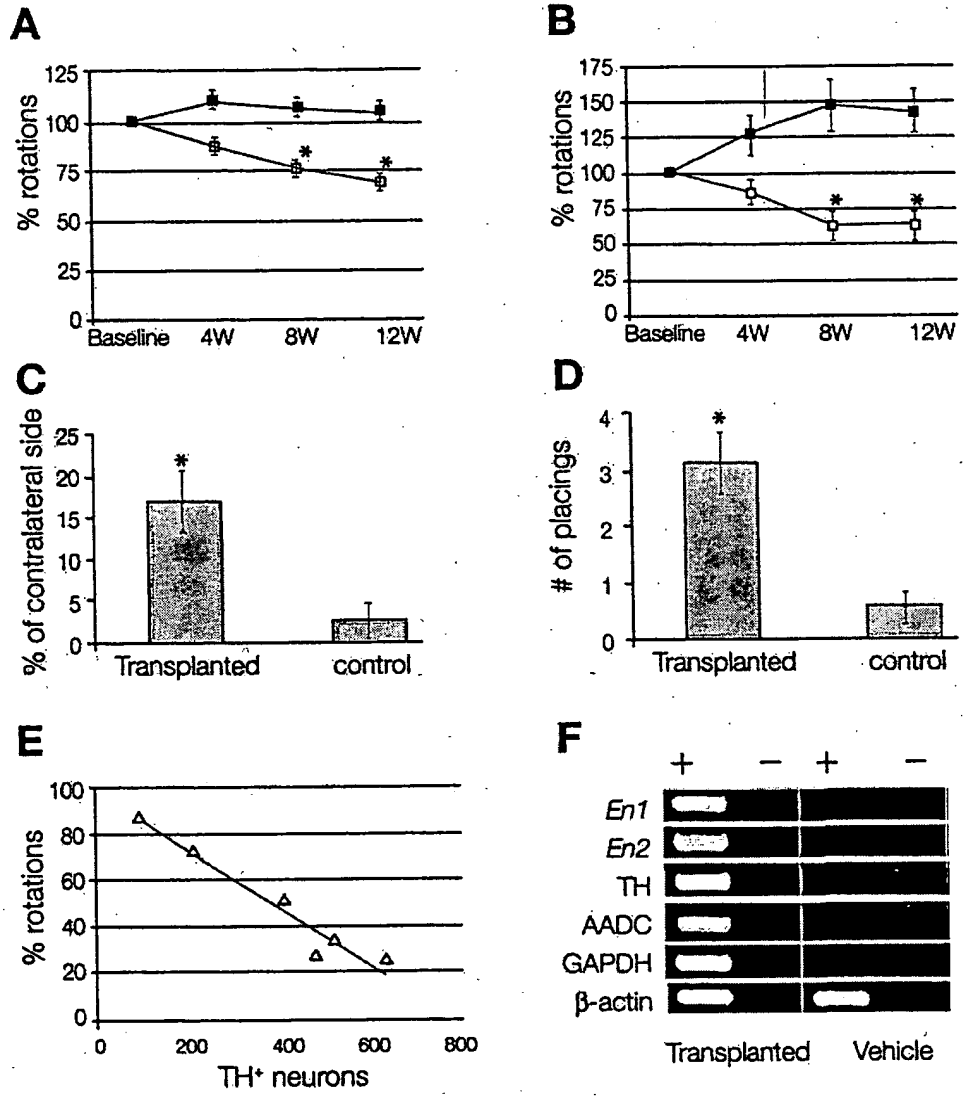

Figure 3. Transplantation of human embryonic stem cell–derived NPs improves motor function in Parkinsonian rats. The number of apomorphine- or D-amphetamine–induced rotations was calculated individually for each rat as a percentage of its performance at baseline. For each time point, the value represents the mean ± standard error percentage of rotations. Rotational behavior that was induced by (**A**) apomorphine, and (**B**) D-amphetamine decreased significantly in transplanted animals, as compared with baseline and control rats. Both (**C**) stepping and (**D**) placing are indicated as the percentage of forelimb stepping adjustments and placing in the lesioned side, as compared with the nonlesioned side. Both tests improved significantly ($p = .0012$ and .0003, respectively) in transplanted rats, as compared with controls. (**E**): The total number of TH^+ neurons in the graft of individual rats correlated with the extent of reduction of amphetamine-induced rotations at 12 weeks following transplantation ($r = -.97$; $p = .001$). (**F**): Reverse transcription polymerase chain reaction was used to analyze striata samples from sphere-grafted and vehicle-grafted animals for the expression of human-specific transcripts of midbrain and dopaminergic neuron markers. The human-specific transcripts (for *En1*, *En2*, TH, L-AADC, and GAPDH) were expressed only by animals that received NPs and were not detected in sham-operated animals. β-actin primers were not human specific. A ± indicates presence or absence of reverse transcriptase. ■, controls; Δ, transplanted group. *$p < .05$, as compared with baseline and controls for the pharmacological tests and with the control group for the nonpharmacological tests. Abbreviations: AADC, acid decarboxylase; GAPDH, glyceraldehyde-3-phosphate; NP, neural progenitors; TH, tyrosine hydroxylase.

in serial sections spanning the entire graft in six brains and adjusted to the full volume of the graft. The grafts generated 389 ± 83 TH⁺ neurons (102–630 TH⁺ neurons per brain), which were 0.18% ± 0.05% of the total number of cells within the graft.

Cells that were decorated with an antibody directed against human dopamine transporter, a specific marker of DA neurons, were identified within the graft and were undetectable in the striatum of medium-grafted controls (Fig. 2R).

To further confirm the expression of human dopaminergic neuronal markers in the transplanted brains, we performed RT-PCR analysis of striatal samples from vehicle-grafted ($n = 2$) and NP-grafted ($n = 3$) rats. Human-specific transcripts of midbrain and DA neuron markers were expressed only in samples from NP-grafted animals (Fig. 3F). Expression of the transcripts was found only on the transplanted side, and not on the nonlesioned side of the same animals (not shown).

Given the potential of ES cells to generate teratomas after transplantation, we evaluated the percentage of proliferating cells within the grafts. At 24 hours post-transplantation, the grafts were heavily stained with the neural precursor cell marker nestin (Fig. 2S). At that time, the majority (64.5% ± 0.9%) of cells within the graft were in a proliferative state, as indicated by their immunoreactivity with anti-PCNA (Fig. 2T) and anti-ki67 (not shown). At 12 weeks, there were very rare PCNA⁺ cells (0.2% ± 0.05%; Fig. 2U). In addition, serial H&E-stained sections covering the entire brain did not reveal teratomas or any other tumor formation in transplanted rats.

Functional Recovery of Sphere-Grafted Parkinsonian Rats

Pharmacological-induced rotational behavior was measured in rats that were transplanted either with spheres or with medium at 2 weeks (baseline), 4 weeks, 8 weeks, and 12 weeks after engraftment. Two rats in which no graft was found did not exhibit any improvement in motor function and were excluded from the analysis. In transplanted rats ($n = 19$), apomorphine-induced rotations decreased in the transplanted group from an average of 624 ± 50 times per hour at baseline to 423 ± 36 rotations per hour at 12 weeks (31% decrease, $p = .0015$; Fig. 3A). The control group of rats ($n = 17$) rotated 567 ± 41 times per hour at baseline and 571 ± 27 after 12 weeks. Amphetamine-induced rotations decreased in the transplanted group of rats ($n = 10$) from 607 ± 63 per hour at baseline to 334 ± 41 per hour at 12 weeks (45% decrease, $p = .001$; Fig. 3B). Amphetamine-induced rotations increased in the control group ($n = 10$) from 480 ± 66 times per hour to 571 ± 74 rotations per hour at 12 weeks. The total number of TH⁺ neurons in the engrafted striatum, at 12 weeks after transplantation, was compared with the degree of behavioral improvement ($n = 6$ animals; Fig. 3E). A significant correlation was found between the number of TH⁺ neurons and the degree of improvement in amphetamine-induced rotational behavior ($r = -.97, p = .001$).

Stepping adjustments [9] and forelimb placing [10] are nonpharmacological tests, which may provide a more direct measure of motor deficits that are analogous to those found in human Parkinson's disease [7]. Stepping and placing were examined at baseline (2 weeks) and at 12 weeks after transplantation. At 2 weeks, the transplanted rats ($n = 11$) did not make any stepping or placing movements on the lesioned side. At 12 weeks, there was a significant improvement in both nonpharmacological tests and in baseline and control rats ($n = 10$; Fig. 3C–D).

Discussion

hESCs attract remarkable interest due to their potential value for cell therapy in Parkinson's disease. Here, we present the first histological and behavioral analysis following transplantation of hESC-derived neural progeny in a rat model of Parkinson's disease.

We used a simple protocol to direct the differentiation of hESCs into highly enriched cultures of NPs. The NPs expressed transcripts of key regulatory genes of midbrain development, as well as markers of DA neurons, supporting their potential to differentiate into midbrain DA neurons.

After transplantation, a significant functional effect was apparent, as shown by four different behavioral tests. While the pharmacological tests represent asymmetry in dopamine release and in postsynaptic sensitivity to dopamine, the nonpharmacological tests indicate reduced motor activity, which is more closely relevant to the motor manifestations of human Parkinson's diseases. The improvement of behavior was correlated with the demonstration of transplant-derived DA cells, as indicated by immunofluorescence stainings and RT-PCR. At the RNA level, human-specific transcripts of key regulatory genes of midbrain development, as well as markers of DA neurons, were observed in brain samples from the location of the graft. At the protein level, human cells decorated with antibodies against the dopamine neuron–specific marker DAT were observed within the grafts.

It has been shown that low numbers of TH⁺ cells can bring about functional recovery in the rat Parkinson model. Transplantation studies of in vitro–expanded rat mesencephalic precursors demonstrated that 1,200 grafted DA cells induced partial functional recovery [14]. Studies of primary fetal rat mesencephalic grafts demonstrated that 1,200 dopamine neurons induced complete recovery of amphetamine-induced rotational behavior 6 weeks after transplantation, and that about 400 TH⁺ neurons were required for a 50% reduction in motor asymmetry [15]. In our study, the hESC-derived grafts

gave rise to 389 ± 83 TH^+ cells, which is consistent with the partial functional effect observed. In addition, the degree of functional improvement correlated with the TH^+ cell counts.

Several studies have suggested that behavioral recovery following cell transplantation may be related to a trophic effect on dying host dopamine neurons [16]. It is possible that trophic effects may have partly contributed to the behavioral recovery that we observed. Further studies are required to confirm that hESCs can differentiate into DA neurons with phenotypes, functionality, and interactions with host neurons that are identical to those of authentic midbrain DA neurons, and to evaluate their potential trophic effect.

Transplantation of neural precursors into the brain at the acute stage of stroke or in inflammatory disease results in their migration toward the lesion and differentiation into the type of cells that were injured [17]. Here, the 6-hydroxydopamine lesion induces neuronal degeneration that lacks components of active inflammation or a regenerative process in the brain. The host tissue probably could not direct the transplanted NPs to acquire a DA fate and the proportion of graft-derived TH^+ cells in vivo was low, which is similar to their spontaneous occurrence in vitro. Therefore, commitment of the human cells to a DA fate, prior to their transplantation, is a prerequisite for obtaining a larger number of graft-derived DA neurons. Also, it will be important to define the specific developmental stage of such committed precursors that will result in optimal survival, functional integration, and behavioral effects.

Transplantation of low numbers of dissociated undifferentiated mouse ES cells into Parkinsonian rats gave rise to a larger number of TH^+ neurons than seen in this study [6]. The reason for this difference is unknown. It may be related to the transplantation of dissociated cells in the mouse ES cell study, as opposed to clumps described in this study, to differences in the stage of development of transplanted cells, or to differences in the biology of cells originating from different species.

The undifferentiated ES cells that were transplanted in the mouse study gave rise to teratomas in a high percentage of the animals. Our cultures of committed NPs did not include undifferentiated hESCs, as suggested by the lack of expression of Oct4. At 12 weeks after transplantation, the engrafted NPs ceased to proliferate, and teratomas or non-neural tissue were not observed. Since a comprehensive search for hESC-derived mesodermal and endodermal progeny was not performed, the differentiation of transplanted human cells to non-neural lineages cannot be ruled out. Thus, while these results are encouraging, additional extensive long-term studies are required to determine the safety of hESC-derived neural progeny transplantation and to rule out potential hazards such as tumor formation or the development of non-neural cells.

Conclusions

In conclusion, we show for the first time partial functional recovery following transplantation of hESC-derived NPs in an experimental model of Parkinson's disease. The transplanted NPs were not directed by the host striatum to differentiate into DA neurons; therefore, induction of their differentiation into a DA fate, prior to transplantation, may potentially improve the functional effect that we have observed [7, 18]. Our observations encourage further efforts that may eventually allow the use of hESCs for the treatment of Parkinson's disease.

Acknowledgments

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EXHIBIT J

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Gene expression in human neural stem cells: effects of leukemia inhibitory factor

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Abstract

Human neural precursor cells grown in culture provide a source of tissue for drug screening, developmental studies and cell therapy. However, mechanisms underlying their growth and differentiation are poorly understood. We show that epidermal growth factor (EGF) responsive precursors derived from the developing human cortex undergo senescence after 30–40 population doublings. Leukemia inhibitory factor (LIF) increased overall expansion rates, prevented senescence and allowed the growth of a long-term self renewing neural stem cell (ltNSC^{ctx}) for up to 110 population doublings. We established basal gene expression in ltNSC^{ctx} using Affymetrix oligonucleotide microarrays that delineated specific members of important growth factor and signaling families consistently expressed across three separate lines. Following LIF withdrawal, 200 genes showed significant decreases. Protein analysis confirmed LIF-regulated expression of glial fibrillary acidic protein, CD44, and major histocompatibility complex I. This study provides the first molecular profile of human ltNSC^{ctx} cultures capable of long-term self renewal, and reveals specific sets of genes that are directly or indirectly regulated by LIF.

Keywords: epidermal growth factor, leukemia inhibitory factor, microarray, neural stem cells, neurosphere.

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Multi-potent precursor cells derived from the developing or adult brain can be isolated in culture and expanded in the presence of mitogens (for review, see McKay 1997; Gage 2000). In some of these techniques, the cells are grown in free-floating aggregates termed 'neurospheres' (Reynolds *et al.* 1992). Neurospheres have also been generated from human post-mortem fetal tissues using a variety of growth factors and culturing methods (Svendsen *et al.* 1996; Chalmers-Redman *et al.* 1997; Svendsen *et al.* 1998; Carpenter *et al.* 1999; Vescovi *et al.* 1999). Neurospheres have great potential for possible cell therapy applications and as a model for mechanisms of human development and disease. For example, cells derived from neurospheres can migrate and integrate after transplantation into the developing or adult brain (Svendsen *et al.* 1996; Svendsen *et al.* 1997a; Flax *et al.* 1998; Fricker *et al.* 1999; Rosser *et al.* 2000; Qu *et al.* 2001; Ostenfeld *et al.* 2002b; Wu *et al.* 2002). We have recently shown that neurospheres isolated from Down syndrome fetal tissue have specific genetic deficits and undergo reduced neurogenesis at later passages (Bahn *et al.* 2002). However, these intriguing findings underscore the fact that very little is known about the temporal pattern of growth in these cultures or the exact cellular composition of neurospheres.

Where clonal analysis has been performed, a number of cells within rodent neurospheres have been shown to self

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Abbreviations used: BDNF, brain-derived neurtrophic factor; BMP, bone morphogenetic protein; BrdU, 5-bromo-2′-deoxyuridine; CNTF, ciliary neurotrophic factor; DMEM, Dulbecco's modified Eagle's medium; EFN, ephrin; EGF, epidermal growth factor; EST, expressed sequence tag; FGF-2, basic fibroblast growth factor; FGFR, FGF receptor; GDNF, glial-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; IL, interleukin; LIF, leukemia inhibitory factor; ltNSC^{ctx}, long-term cortical neural stem cell; MHC-I and II, major histocompatibility complex I and II; NSC, neural stem cell; NT, neurotrophin; PBS, phosphate-buffered saline; PD, population doubling; PDGFR, platelet-derived growth factor receptor; PSF, penicillin-streptomycin-amphotericin; SDS, sodium dodecyl sulfate; STAT1 and STAT3, signal transducer and activator of transcription 1 and 3.

renew, and to generate neurons, astrocytes and oligodendrocytes; these cells have been termed neural stem cells (NSC) (Reynolds and Weiss 1992; Morshead *et al.* 1994). Cells from human neurospheres have proven more difficult to clone, although, where this has been achieved, some of these cells have also been shown to be multipotent NSC (Johansson *et al.* 1999; Vescovi *et al.* 1999; Suslov *et al.* 2002). The remainder of the cells within the neurosphere may represent more restricted progenitor cells, or simply a subtype of stem cell that cannot survive and generate new spheres under clonal conditions. Nestin, an intermediate filament protein, is expressed within the neuroepthelium during early development (Tohyama *et al.* 1992) and is present in most cells within growing neurospheres (Vescovi *et al.* 1993). However, nestin expression does not distinguish the 'sphere-forming stem cell' from other cells. Recently, a few potential candidate proteins, including nucleostemin (Tsai and McKay 2002) and AC133 (Uchida *et al.* 2000), have been described. In particular, AC133 is present in most cells within neurospheres following their initial isolation and has been used to sort presumptive human neural stem cells from other differentiated cells in primary fetal tissue (Uchida *et al.* 2000). Recent advances in oligonucleotide microarray technology that allow large-scale screening of gene expression in stem cells from a wide range of tissues may lead to the discovery of new stem-cell markers (Geschwind *et al.* 2001; Ivanova *et al.* 2002; Ramalho-Santos *et al.* 2002). However, a detailed description of genes constitutively expressed in human neurospheres is not currently available.

A major challenge in describing genes expressed in neurospheres is selecting exactly which type of neurosphere to analyze. Isolated human NSC are heterogeneous and have varied patterns of gene expression (Suslov *et al.* 2002). In addition to cellular heterogeneity within the neurosphere, there is also evidence that rodent and human neurospheres are regionally specified. When isolated from different regions of the developing or adult brain, NSC display very different characteristics with regard to growth, differentiation and gene expression (Hitoshi *et al.* 2002; Ostenfeld *et al.* 2002a). Similar regional specificity has recently been shown for stem cells isolated from different regions of the neural crest (Bixby *et al.* 2002). Furthermore, the neurospheres derived from rat brain produce progressively fewer neurons, but an increasing number of glia as passage time increases (Ostenfeld *et al.* 2002a). Finally, there are significant differences in neurosphere growth between species such as rat and mouse (Svendsen *et al.* 1997b). All of these factors must be taken into account when attempting to standardize culture growth conditions and basal gene expression in neurosphere cultures.

One of the most important aspects of maintaining dividing cells of any type in culture is the composition of the growth medium. In particular, growth factors and cytokines have been crucial in determining both rate of growth and inhibition of differentiation of neural stem-cell cultures. Leukemia inhibitory factor (LIF) is a member of a family of interleukin-6 (IL-6) cytokines that also includes IL-11, ciliary neurotrophic factor (CNTF), oncostatin M, and cardiotrophin-1. These factors signal through a receptor complex made up of unique components specific for the particular cytokine and a common receptor subunit, gp130. LIF is known for its ability to maintain primitive mouse embryonic stem cells isolated from the inner cell mass of the embryo in an undifferentiated state (Smith *et al.* 1988). Cytokines also play an important role in the nervous system, (for review, see Patterson 1992; Auernhammer & Melmed 2000; Turnley and Bartlett 2000). For example, LIF inhibits the terminal differentiation olfactory receptor neurons (Moon *et al.* 2002) and is also known to affect the maturation of sympathetic neurons (Nawa *et al.* 1991). Of interest to us, was the observation that LIF can increase the longevity of basic fibroblast growth factor (FGF-2) responsive cells within human neurospheres derived from the cortex, although it had no effect on the same cultures grown in epidermal growth factor (EGF) (Carpenter *et al.* 1999). In another study, CNTF has been shown to maintain forebrain murine neurospheres in an undifferentiated state by suppression of glial cell fate restriction (Shimazaki *et al.* 2001). In the current study, we explored the effects of LIF on human neurospheres derived from fetal cortex and grown in EGF, combined with a novel method of passaging which maintains cell-to-cell contact (Svendsen *et al.* 1998). We show that LIF is required for the long-term growth of these EGF responsive cells ($ltNSC^{ctx}$) after more than 30 population doublings (PD) and that these neurospheres show a very stable profile, dividing steadily for up to 110 PD. We also describe for the first time basal gene expression in these cells using the U133 Affymetrix oligonucleotide microarray to assess 33 000 known genes and expressed sequence tags (ESTs). Finally, we identify specific genes that change when LIF is withdrawn from these cultures, which may be important for long-term neural stem-cell self renewal in this system.

Experimental procedures

Tissue collection

Human embryonic tissue (between 8 and 13 weeks post conception) was collected following routine terminations of pregnancy. All embryonic tissue was kindly provided by Dr Eric Jauniaux, Department of Obstetrics and Gynaecology, University College, London. Full ethical approval had been granted by the Local Research Ethics Committee, University College Hospital, London. The methods of collection conform with the arrangements recommended by the Polkinghorne Committee and NIH for the collection of such tissues and to the guidelines set out by the United Kingdom Department of Health and as well as the University of Wisconsin.

Cell culture

Cortical progenitors were isolated from embryonic CNS and induced to proliferate as free-floating neurospheres as previously

detailed (Svendsen *et al.* 1998; Ostenfeld *et al.* 2002a). Freshly dissected tissue was dissociated in trypsin and seeded into T75 flasks at a density of 200 000 cells/mL of serum-free medium consisting of 70% Dulbecco's modified Eagle's medium (DMEM), 30% Hams F12, 1% penicillin/streptomycin/amphotericin B (PSF) supplemented with 2% B27 (v/v) (Life Technologies, Rockville, MD, USA), 20 ng/mL EGF (Sigma, St Louis, MO, USA), 20 ng/mL FGF-2 (R & D Systems, Minneapolis, MN, USA). Heparin (5 μg/mL) was added to stabilize the FGF-2 and increase initial growth rates (Caldwell and Svendsen 1998). All cultures were maintained in a humidified incubator (37°C, 5% CO_2 in air) and half the growth medium was replenished every 4–5 days. Neurospheres were passaged every 14 days by sectioning of neurospheres into 200-μm sections that were seeded into fresh growth medium at a density equivalent of 200 000 cells/mL. At 2 weeks after the first passage, cells were switched to basal media containing 1% (v/v) N2 (Life Technologies) and 20 ng/mL EGF. At 20 weeks of growth, 10 ng/mL LIF (Chemicon, Temecula, CA, USA) was added to the cultures. In cases where LIF was withdrawn, neurospheres were collected, medium removed, and cells washed two times with 1% N2 supplemented with 20 ng/mL EGF, and cultured into the same.

Growth studies

Individual spheres (approximately 0.30 mm in diameter) for each treatment were transferred to a single well of a 96-well plate ($n = 8$) containing 200 μL of growth medium supplemented with the appropriate growth factor combination. Diameter measurements were taken every 4 days using a lens-mounted micrometer. The volume of each sphere was calculated as an index of cell number as previously described in detail (Svendsen *et al.* 1998). [^{3}H]thymidine incorporation was assayed as described (Svendsen *et al.* 1998). For BrdU incorporation assays, ltNSC^{cbx} were pulsed with 0.2 μM 5-bromo-2′-deoxyuridine (BrdU, Sigma, St Louis, MO, USA) for 14 h before disassociation and were plated to poly L-lysine, laminin coated coverslips for 60 min before fixation as described below. Following fixation, cells were stained with 1 : 300 anti-BrdU (Roche, Indianapolis, IN, USA) according to manufacturer's instructions.

Cell counts

Cell counts were performed using a Nikon fluorescence microscope (40 × objective) and Metamorph Imaging software (Universal Imaging Corporation, Downington, PA, USA). Quantification of cells was based on counting the number of Hoechst-stained nuclei and the specified immuno-markers in at least five independent fields (total area >25 mm^2) from a minimum of three coverslips. In the case of the differentiated neurospheres, at least five random fields were analyzed from the monolayer surrounding the plated sphere. All data represent means across three separate human neural stem cell lines derived from the cortex.

Microarray analysis

Samples for gene array analysis were prepared from total RNA and array analysis was performed as described (Li and Johnson 2002). Fragmented cRNA (15 μg) was hybridized 16 h at 45°C to either the HG-U95a array for the preliminary time course study or the HG-U133 array set for the LIF-dependent gene study (Affymetrix, Santa Clara, CA, USA). After hybridization, the gene chips were automatically washed and stained with streptavidin-phycoerythrin by using a fluidics station. Finally, probe arrays were scanned at 3-μm resolution using the Genechip System confocal scanner made for Affymetrix by Aligent. Affymetrix Microarray Suite 4 (preliminary study) or 5 (LIF- dependent gene study) were used to scan and analyze the relative abundance of each gene derived from the average difference of intensities. Analysis parameters used by the software were set to values corresponding to moderate stringency (SDT = 30, SRT = 1.5). The threshold values to determine the present (P) or absent (A) call were set as follows: $\alpha 1 = 0.05$, $\alpha 2 = 0.065$, $\tau = 0.015$. Fluorescence intensity was measured for each chip and normalized to the average fluorescence intensity for the entire chip. Output from the microarray analysis was merged with the Unigene or GenBank descriptor and stored as an Excel data spreadsheet. The definition of increase (I), decrease (D), or no change (NC) of expression for individual genes was based on ranking the Difference Call from three comparisons (3 × 1), namely, No Change = 0, Marginal Increase/Decrease = 1/−1, Increase/Decrease = 2/−2. The final rank referred to summing up the three values corresponding to the Difference Calls and the value varied from −6 to 6. The cut-off value for the final determination of Increase/Decrease was set as 3/−3. Evaluation of the reproducibility of paired experiments was based on calculation of the coefficient of variation (CV) (SD/mean) for fold change (FC). The CV of FC must be less than or equal to 1.0. Finally, only genes with a FC over 1.5 were considered significant. These cut-off values gave a conservative estimate of the numbers of genes whose expression levels differ between samples. Gene categorization was based on a literature review.

Immunocytochemistry

For acute studies, neurospheres were dispersed into a single cells suspension with Accutase (Innovative Cell Technologies, San Diego, CA, USA) for 10 min at 37°C and 30 000 cells were plated onto 0.01% poly L-lysine and 10 μg/mL laminin-coated glass coverslips in 24-well plates and incubated for 60 min at 37°C. The plating media consisted of 2% B27 in 70% DMEM, 30% Hams F12, 1% PSF. Cells were fixed with 4% paraformaldehyde for 20 min or ice-cold methanol for 5 min and washed with phosphate-buffered saline (PBS). For differentiation studies, whole spheres were plated directly in 2% B27 plating medium onto 0.01% poly-L-lysine and 10 μg/mL laminin-coated glass coverslips in 24-well plates. After 14 days, the spheres were fixed with 4% paraformaldehyde for 20 min and washed with PBS. Fixed cell cultures were blocked with 5% normal goat serum and 0.2% Triton X-100 (omitted for cell surface proteins) and processed for immunocytochemistry with primary antibodies to β-tubulin III (monoclonal, 1 : 5000, Sigma), glial fibrillary acidic protein (GFAP) (polyclonal, 1 : 1000, Dako, Carpenteria, CA, USA), nestin (monoclonal (hNestin-10C2) or polyclonal 1 : 200, gift of Eugene Major, National Institute of Neurological Disorders and Stroke), W6/32 (monoclonal, 1 : 10, gift of Etienne Joly University of Cambridge). Following rinsing with PBS, the cells were incubated for 30 min with secondary antibodies conjugated to either fluorescein or rhodamine. Hoechst 33258 (1 : 10 000 in PBS) was added for 5 min after completion of the secondary antibody incubation as a nuclear stain. All cell-count data was expressed as means ± SEM and were analyzed using one-way ANOVA with Newman–Keuls post hoc test.

Preparation of cell-free lysates and immunoblotting

Neurospheres were isolated and suspended in 1 mL of lysis buffer (Mandal *et al.* 2001) expelled through a 28-gauge needle, incubated for 15 min on ice, centrifuged for 15 min at 10 000 *g* at 4°C and stored at −80°C. Protein concentration was determined by a detergent-compatible modified Bradford assay (Bio-Rad, Richmond, VA, USA). Protein from cell-free lysates (10 μg) was separated on 4–15% gradient SDS-polyacrylamide gels, electro-transferred to immobilon membrane, blocked with 5% dry milk in Tris-buffered saline, probed with anti GFAP (polyclonal, 1 : 1000, Dako) for 2 h, and then incubated with a secondary antibody (anti-rabbit IgG conjugated to peroxidase, 1 : 2000, Promega, Madison, WI, USA). Antibody staining was visualized using chemiluminescence kit from Amersham (Piscataway, NJ, USA).

Results

LIF is essential for growth of EGF responsive NSC

Neurospheres were generated from two human fetal cortex samples, K052 (11 weeks post conception) and K054 (13 weeks post conception). The cells were grown for 4 weeks in media supplemented with EGF and FGF-2, followed by culture in EGF alone and passaged by a novel 'chopping' method that does not require trypsin or mechanical dissociation and where cell/cell contact was consistently maintained (Svendsen *et al.* 1998). These cultures grew steadily as neurospheres with a PD time of approximately 4.5 days. However, after 30 weeks of culture, the PD time had increased to more than 10 days in EGF alone, indicating a significant slow down in growth, and, by 40 weeks of culture, no further expansion occurred (data not shown). This pattern was also found with a number of other cortical cultures, and, although absolute growth rates varied, all lines senesced before 30 weeks of growth or approximately 40 PD.

In order to establish whether cytokines could increase growth rates and prevent the senescence seen with EGF alone, the effects of adding LIF, CNTF or IL-6 to neurospheres were examined. Following 20 weeks of culture in EGF, individual neurospheres were isolated, exposed to LIF, CNTF or IL-6 at 10 ng/mL for 18 days, and the changes in sphere volumes were measured as a growth index. LIF significantly increased growth rates of K052 and K054 by approximately 90% over EGF alone (Figs 1a and b). CNTF also increased growth rates by 78% but IL-6 had no significant effect. In a second experiment, neurospheres were grown for 20 weeks in EGF, exposed to LIF, CNTF or IL-6 at 10 ng/mL for 17 days, pulsed with ^{3}[H]thymidine for 24 h, and radioactivity was measured at day 18. LIF increased the number of dividing cells 2-fold as determined by ^{3}[H]thymidine incorporation (Fig. 1c). We next asked whether cultures maintained in EGF/LIF became dependent upon LIF for continual growth. Neurospheres generated from K052, K054 and an additional line K066 (8 weeks post conception) were grown for additional 30 weeks in EGF and

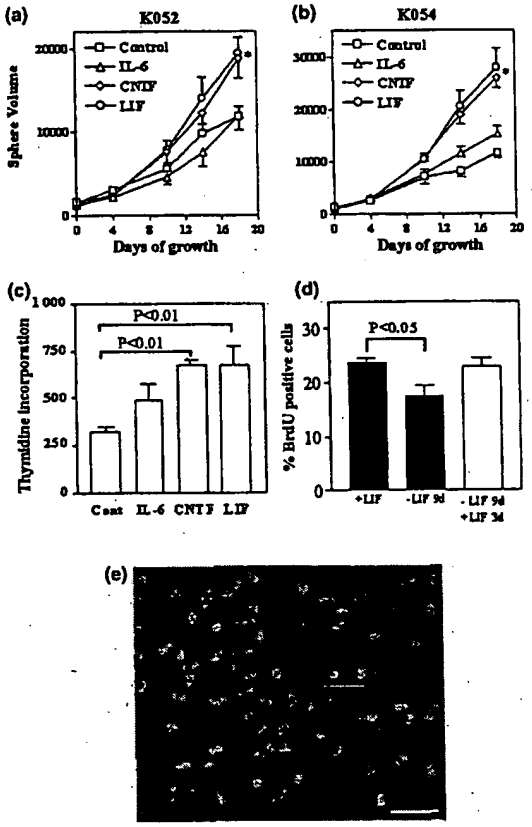

Fig. 1 LIF increases growth rate of EGF responsive ltNSC^{ctx}. ltSC^{ctx} were grown as neurospheres for 20 weeks in defined media supplemented with 20 ng/mL EGF. LIF (○), CNTF (◇) and IL-6 (△) were added at 10 ng/mL to individual spheres for 18 days and sphere volume measurements were taken every 4 days and compared with control (□) for two cell lines, K052 (a) and K054 (b). EGF responsive ltNSC^{ctx} were grown for 18 days in EGF-containing media supplemented with LIF, CNTF, or IL-6 at 10 ng/mL and then pulsed for 24 h with 0.5 μCi/mL [^{3}H]thymidine (c). ltNSC^{ctx} cultures (K052, K054, K066) were grown for a total of 50 weeks in media supplemented with EGF for the entirety of culture and LIF supplementation starting at 20 weeks, withdrawn from LIF for 9 days followed by re-exposure to LIF for 3 days. Cells were pulsed with 0.2 μM BrdU for 14 h, dissociated and plated for 1 h before fixation and immuno-staining with anti-BrdU (d). Whole neurospheres from ltNSC^{ctx} grown long-term in EGF/LIF were plated to poly L-lysine/laminin coated coverslips and differentiated for 14 days then visualized with anti-TuJ1 (red) and Hoechst-labeled nuclei (blue) (e). The asterisk denotes $p < 0.01$.

LIF following the initial culturing for 20 weeks in EGF alone. LIF was then withdrawn for 9 days and then re-applied for 3 days. All cultures were pulsed with 0.2 μM BrdU for 14 h before dissociation, plate-down for 60 min, fixation, and immuno-staining with anti-BrdU. When LIF was withdrawn, cells within the neurospheres reverted to

a slower growth rate within 9 days based on BrdU incorporation (Fig. 1d). Within 3 days re-application of LIF, growth rates returned to pre-withdrawal levels. LIF was able to extend the *in vitro* culture time of these cells for up to 70 weeks (110 PD) and preserved the original doubling time of 4.5 days throughout this time period (data not shown). To examine the differentiation potential of these long-term cultures maintained in EGF and LIF (ltNSC^{ctx}), individual neurospheres from K052, K054 and K066 (40 PD) were plated to laminin/poly L-lysine coated coverslips for 14 days under differentiating conditions. After fixation and immuno-staining for the neuronal marker β-tubulin III (Tuj1) (Menezes and Luskin 1994), these cultures produced approximately 30% Tuj1 positive cells (Fig. 1e).

The withdrawal of LIF might simply have caused a reduction in growth rates by increasing the differentiation of cells into post-mitotic neurons within the neurosphere. To investigate this possibility, LIF was withdrawn for 9 days from long-term cultures grown in EGF/LIF. The neurospheres were then dissociated to a single-cell suspension and plated for 1 h before fixation and staining for the neuronal marker Tuj1 (Figs 2b and c), and the progenitor cell marker, nestin (Tohyama *et al.* 1992) (Figs 2a and c). Following LIF withdrawal, the number of Tuj1 positive cells (< 2% of the population) and nestin positive cells (> 92% of the population) remained unchanged (Fig. 2d). Together these results suggest that LIF dynamically regulated some aspect of the nestin positive cells which both increased growth rates and allowed long-term self renewal.

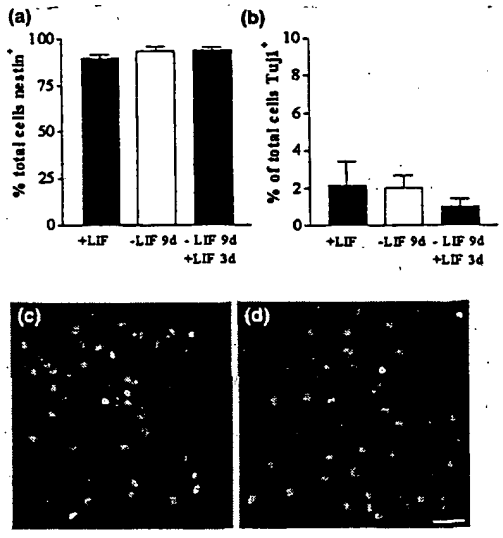

Fig. 2 LIF has no effect on nestin or TuJ1 expression in ltNSC^{ctx}. ltNSC^{ctx} cultures (K052, K054, K066) were grown for a total of 50 weeks in media supplemented with EGF for the entirety of culture and LIF supplementation starting at 20 weeks, withdrawn from LIF for 9 days followed by re-exposure to LIF for 3 days. Whole spheres were dissociated and plated for 1 h before fixation, and then immunostained for nestin (red), Tuj1 (green) and Hoechst-labeled nuclei (blue). The percentages of nestin-positive (a) and TuJ1-positive cells (b) were determined. There were no significant differences in the percentage of labeled cells, or in the type of cell as seen in the photomicrograph of cells grown long-term in EGF/LIF (c) compared with that of cells withdrawn from LIF for 9 days (d). Bar: 50 μm.

ltNSC^{ctx} show stable gene expression over an extended period of time

In order to establish basal gene expression in our culture system, and begin to explore the effects of LIF, we performed a series of oligonucleotide microarray experiments. In a preliminary study, the pattern of gene expression in a single ltNSC^{ctx} line (K066) was compared over time. K066 was grown for 20 weeks in EGF only, and subsequently supplemented with both EGF and LIF. Total RNA was harvested at 34 and 53 weeks of culture, and gene expression profiles were compared using Affymetrix HG-U95a microarray (9670 genes and ESTs). Affymetrix Microarray Suite 4.1 was used to scan and analyze the relative abundance of each gene. The signal output from each gene from the 34-week profile was plotted against the 53-week profile (Fig. 3), and the correlation coefficient (r) was calculated for each comparison. As shown by the very high r value (0.975), the total gene expression levels in ltNSC^{ctx} were stable for approximately 20 weeks in the long-term presence of EGF and LIF.

Basal gene expression in LIF treated ltNSC^{ctx} cultures

To determine which genes might be effected by LIF, we compared 3 ltNSC^{ctx} lines (K066, K052, K054) grown for

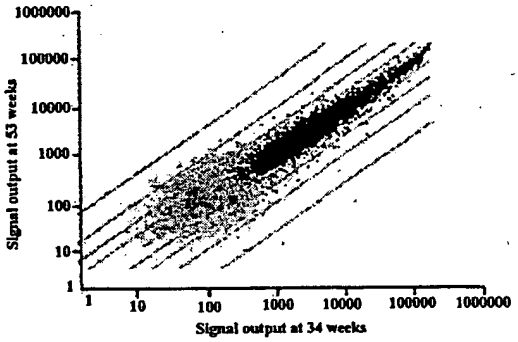

Fig. 3 ltNSC^{ctx} gene expression is stable over time. ltNSC^{ctx} (K066) was grown in media supplemented with EGF for the entirety of culture and LIF supplementation starting at 20 weeks. Total RNA was collected at 34 and 53 weeks and gene expression patterns were compared using HG-95a oligonucleotide array analysis. Lines denote 2-, 4-, 10-, and 20-fold change. Genes absent in both samples are labeled with yellow, genes present only in one sample are labeled in blue, and genes called present in both samples are labeled in red.

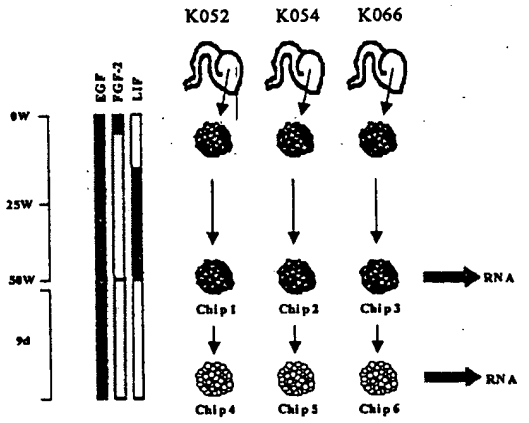

Fig. 4 Schematic describing culture conditions and treatments for three separate ltNSC^{ctx} lines. Neurospheres were generated from three fetal cortex samples, initially grown for 4 weeks in EGF and FGF-2. After 4 weeks, the cultures were switched to media supplemented with EGF alone. At 20 weeks, the cultures were supplemented with EGF and LIF. At 50 weeks, total RNA for oligonucleotide microarray analysis was harvested from these cultures. A second set was withdrawn from LIF but maintained in EGF for 9 days followed by RNA preparation.

20 weeks in EGF only, and thereafter supplemented with both EGF and LIF for an additional 30 weeks (50 weeks total). We expanded the total number of genes to be screened by using the newly available Affymetrix HG-U133 oligonucleotide arrays. This gene array set contains 33 000 expressed genes and expressed sequence tags (ESTs) compared with 9670 genes on the HU-95 array. The experimental design is shown in Fig. 4. Affymetrix Suite 5 was used to analyze the data as described in methods. Gene expression that exceeded the criteria for perfect match/mismatch was called present; gene expression that failed to meet the criteria was called absent. Approximately 40% of the sequences contained on the arrays were called present within long-term EGF/LIF-treated ltNSC^{ctx}, 58% were called absent and 2% were assigned a marginal call. When signal output of each probe set for the one sample was plotted against each of the other samples, the correlation coefficients ranged from 0.923 to 0.983, indicating a great degree of similarity between the samples.

We examined genes that are important in the development of the nervous system as listed in Table 1. Only those genes called present in all three cell lines are shown. As expected, nestin, notch, cyclins, and the EGF receptor were called present. Several genes identified as stem-cell specific (Geschwind *et al.* 2001; Ivanova *et al.* 2002; Ramalho-Santos *et al.* 2002) including Fyn, ephrin B2 (EFN-B2), Slit2, insulin-like growth factor binding protein 2 and AC133 (Uchida *et al.* 2000) were also present in ltNSC^{ctx}. p53, an important determinant of normal cell functioning (Levine *et al.* 1991) was not changed by LIF. Subsets of genes within important families were selectively expressed. For example, members of the important bone morphogenetic protein (BMP) family which is involved in epidermal induction (Munoz-Sanjuan and Brivanlou 2002) and glial fate (Nakashima *et al.* 2001) were selectively expressed. BMP 1, 5 and 15 were called present whereas BMP 2, 3, 6, 8 and 10 were absent. ltNSC^{ctx} did not express the genes for growth factors such as brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), neurotrophin 3 (NT-3), NT-4 or any of the neurogenins; however, there was expression of FGF-2 and its receptor FGFR1, but not FGF-8. There were also some genes we expected to see expressed which were called absent such as musashi1 and the all of the *wnt* family. The full list of genes called absent and present across the three lines can be found in supplementary Excel Table S1 (available online at http://www.waisman.wisc.edu/genechipdata/).

ltNSC^{ctx} from the three lines, were subject to 9 days LIF withdrawal, and the gene expression profiles were compared with the corresponding EGF/LIF cultures (Fig. 4). Using the criteria described in Experimental procedures, we found that 531 genes were decreased and 193 were increased upon LIF withdrawal. A complete listing of genes that show LIF-dependent changes in expression can be found in supplementary Table Excel S2 (available online at http://www.waisman.wisc.edu/genechipdata/). Only 200 genes meeting the requirements of a ranking score of −5/−6 or +5/+6, a fold change > 1.5, and a CV < 0.8 were included in Table 2. Following separation of different gene clusters, clear trends were established with regard to which genes significantly declined following 9 days of LIF withdrawal. A small number of genes were increased following LIF withdrawal (Table 2). These included neurexin I which is a cell surface protein which binds latrotoxin (Geppert *et al.* 1998) and erb-b, the EGF receptor. There were some unexpected results. For example, cystatin C which is required for FGF-2 responsive neural stem cells (Taupin *et al.* 2002) was significantly increased upon LIF withdrawal. Furthermore, the expression of cyclin D1, a putative stem cell specific marker (Geschwind *et al.* 2001; Ivanova *et al.* 2002; Ramalho-Santos *et al.* 2002) was also increased upon LIF withdrawal.

A much larger number of genes were decreased following LIF withdrawal (Table 2). Transcription factors that are associated with the GP130 signaling system, including signal transducer and activator of transcription 1 and 3 (STAT1, STAT3) were modulated by LIF. A number of genes, such as EFN-A1, CD44 (Goodison *et al.* 1999), and glypican (Lander *et al.* 1996) that are involved with regulation of cell shape, movement, and interactions with the extracellular matrix, were also decreased. Neuropeptide Y is implicated in neurogenesis within the olfactory system (Hansel *et al.* 2001) and was reduced by threefold following LIF withdrawal.

Table 1 Important genes for development and stem-cell biology expressed in ltNSC^{ctx}

Classification	Genes expressed in ltNSCctx	Genes not expressed
AC-133	prominin (mouse)-like 1 (PROML1) AC 133	
Achaete/scute	similar to ASCL1	ASCL2
BDNF		brain-derived neurotrophic factor (BDNF)
BMP	bone morphogenetic protein 1 (BMP1); BMP5; BMP15	BMP2; BMP3; BMP4; BMP6; BMP8; BMP10; BMPY
Catenin	catenin (cadherin-associated protein), beta 1 (CTNNB1)	
CD9	CD9 antigen (p24)	
CIITA		MHC class II transactivator (CIITA)
Cyclin	cyclin I (CYC1); cyclin-dependent kinase inhibitor 2 A (p16, CDKN2A); CYCB1; CYCD1; CYCD2 (CCND2); CYCE2; CYCG1; CYCG2; Similar to cyclin-dependent kinase inhibitor 1B (p27, Kip1)	CYCT1 (CCNT1); cyclin-dependent kinase-like 1 (CDKL1); CDKL2; cyclin-dependent kinase 3 (CDK3); CDK6; CDKNI2D (p19); cyclin-dependent kinase inhibitor (p16INK4a); CDKN1C (p57, Kip2); CDKNI2C (p18)
Delta	delta-like-1 protein (DLL1), DLL3; DLK	delta 2
DLX	distal-less homeo box 2 (DLX2); DLX5	DLX4; DLX6
Doublecortin	doublecortin (DCX)	
EDR	Early development regulator 1(EDR1); EDR2	
EGF	Epidermal growth factor (EGF); erb-b	
Engrailed		engrailed homolog 1 (EN1); (EN2)
Ephrin	Ephrin-A1 (EFNA1); EFNB2	EFNA2; EFNA3; EFNA4; EFNA5; EFNB3
		ephrin receptor EPHA3; ligand for eph-related receptor tyrosine kinases (EPLG8)
EPO		erythropoietin (EPO); erythropoietin receptor
FGF	fibroblast growth factor 2 (basic) (FGF2); FGF13; FGFR1; FGFR3; FGFR1 oncogene partner (FOP)	FGF8; FGF9 FGFR2; FGFR4; FGFR5
Frizzled	frizzled (Drosophila) homolog 1 (FZD1); FZD2; FZD3; FZD6; FZD; FZD8; Fritz; frizzled-related protein 1; frizzled-related protein (FRZB); secreted frizzled-related protein 4	secreted frizzled-related protein 2; FZD4; FZD5; secreted frizzled-related protein 5 (SFRP5); FZD9; FZD10; membrane-type frizzled-related protein MFRP
FYN	FYN; c-syn; tyrosine kinase p59fyn(T)	FYN-binding protein (FYB-120130)
GCM		glial cells missing (Drosophila) homolog b (GCMB)1
GDNF		glial cell derived neurotrophic factor (GDNF)
		GDNF family receptor alpha 1 (GFRA1)
HES	Hairy enhancer-of-split related with YRPW motif 2 (HEY2); HES1, HES4	HES2; hypothetical protein HES6; HES7; HEY1; HES- related repressor protein 1 HERP1
HIF	hypoxia-inducible factor 1, alpha (HIF1A); hypoxia- inducible protein 2 (HIG2)	
ID	3′ part of the gene for Inhibitor of DNA binding (ID1)1; 5′ end of the ID1 gene; inhibitor of DNA binding 2 (ID2); ID4 and part of an alternatively spliced novel gene	ID3
IGF	Insulin-like growth factor binding protein 2 (IGFBP2); IGF1 receptor (IGF1R); IGF2R; IGFBP5; IGFBP7	IGF1; IGF2; IGFBP1; IGFBPR3; IGFBP4
IL-6 related	leukemia inhibitory factor receptor (LIFR); interleukin 6 signal transducer (gp130); oncostatin M receptor	LIF; ciliary neurotrophic factor (CNTF)
ISL		ISL1 transcription factor, LIM homeodomain (Islet-1)
Jagged	Jagged 1(JAG1); JAG2	
JAK		Jak2 kinase (JAK2); JAK3B splice variant; JAK binding protein
Laminin	laminin, gamma 1(LAMC1); LAMB2; LAMA4	
LYN	Lyn B protein, v-yes-1 oncogene homolog; Lyn	
MAF	v-maf oncogene family, protein G (MAFG); MAFC MAFF	MAFK; v-maf; Kreisler (mouse) maf-related leucine zipper homolog (KRML)
MAP	protein kinase MEKK2b; MAP kinase kinase MEK5c; MEK partner 1 (MP1)	MAP kinase kinase MEK5b

Table 1 (Continued)

Classification	Genes expressed in ltNSCctx	Genes not expressed
Meis	Meis1 (mouse) homolog (MEIS1); TALE homeobox Meis2e; Meis3	
Musashi1		Musashi (Drosophila) homolog 1 (MSI1)
Nestin	nestin	
NeuroD		neurogenic differentiation 1 (NEUROD1); NEUROD4; NDRF gene for neuroD-related factor
Neurogenin		neurogenin 1 (NEUROG1); NEUROG 2; NEUROG3
Neurotrophin		neurotrophin 3 (NTF3); NTF4/5
NKX		NK-2 (Drosophila) homolog B (NKX2B); NKX2.8; NKX3.1; NKX6A
NOGO	Nogo-A protein short form; nogo receptor (NOGOR)	
Notch	Notch (Drosophila) homolog 2 (NOTCH2); NOTCH3	NOTCH1; NOTCH4
NrCAM	neuronal cell adhesion molecule (NRCAM)	
NPTX	neuronal pentraxin I (NPTX1)	NPTX2; DNAL4, neuronal pentraxin receptor and CBX6
Numb	NUMB protein	
OCT		octamer binding transcription factor 1 (OTF1); Oct2; Oct6; OCT11
OTX		ESTs highly similar to OTX1; orthodenticle (Drosophila) homolog 2 (OTX2)
p53	p53 cellular tumor antigen	
Patched		patched (Drosophila) homolog (PTCH); PTCH2
PDGF	platelet-derived growth factor alpha polypeptide (PDGFA); PDGFC; PDGFRA	PDGFRB
PAX	paired box gene 6 (PAX6)	PAX3; PAX4; PAX8
PROX		prospero-related homeobox 1 (PROX1)
PSEN	presenilin (PSEN1); presenilin 2 (PSEN2)	
PTEN		phosphatase and tensin homolog (PTEN)
PTX	pentaxin-related gene, rapidly induced by IL-1 beta (PTX3)	
RA	retinoic acid repressible protein (RARG-1;) retinoic acid- and interferon-inducible protein (58 kDa)	retinoic acid receptor, alpha (RARA); RARB; RAR,gamma zinc finger protein 42 (myeloid-specific retinoic acid-responsive, ZNF42)
Reelin		reelin (RELN)
SHH		sonic hedgehog (Drosophila) homolog (SHH)
Slit	**neurogenic extracellular slit protein Slit2 (SLIT2)**	SLIT1; SLIT3
SOX	SRY (sex determining region Y)-box 3 (SOX3); SOX9; SOX11	SOX1; SOX2; SOX5; SOX13; SRY SOX14; SOX18; SOX 20; SOX21; SOX22; SOX29
Sprouty	sprouty (Drosophila) homolog 1 (SPRY1); SPRY2; **SPRY4**	
STAT	**signal transducer and activator of transcription 1 (STAT1)**; STAT2; **STAT3**; STAT5B; suppressor of cytokine signaling-2 (SOCS-2); SOCS4; protein inhibitor of activated STAT (PIASx-β)	STAT5A; STAT6; SOCS-1; SOCS-3; PIASY; PIAS3
TCF	transcription factor 6-like 1 (mitochondrial transcription factor 1-like) (TCF6L1); **TCF3**; TCF4; TCFL5; **TCF8**; e12 protein (E2A) transcription factor 3 (E2A immunoglobulin enhancer binding factors); TCF3 (E2A) fusion partner (in childhood Leukemia) (TFPT)	TCF7; TCF15; TCF17; TCF21
TERT		Telomerase (TERT)
TGF	transforming growth factor (TGF) beta-induced transcription factor 2; TGFBR2	TGFA; -beta 3 (TGF-beta3); TGFB2; TGF-b superfamily receptor type I (TGFBR1)
TRK	neurotrophic tyrosine kinase, receptor, type 2 (NTRK2); trkC human, brain; NTRK3	NTRK1
VEGF	vascular endothelial growth factor B (VEGFB)	
Vimentin	vimentin	

Table 1 (Continued)

Classification	Genes expressed in ltNSCctx	Genes not expressed
WNT		wingless-type MMTV integration site family, member 1 (WNT1); WNT3; WNT4; WNT5B; WNT6; WNT7a; WNT8b; WNT8d; WNT10a; WNT10b; WNT16
Marginal call		
IL-6 related	ciliary neurotrophic factor receptor (CNTFR)	
laminin	laminin, alpha 2 (merosin, congenital muscular dystrophy) (LAMA2)	
Cyclin	cyclin-dependent kinase 2; cyclin M1 (CNNM1)	

The expression of selected genes that are important in development and stem-cell biology was segregated in those genes that had detectable expression in ltNSC^{ctx} grown long term in EGF/LIF and those genes with no detectable expression and receiving an absent call. Genes that are highlighted in bold exhibit LIF dependent changes.

Table 2 LIF-dependent changes in ltNSC^{ctx}

Genes decreased upon LIF withdrawal	CV	Fold change
Immune response		
MHC class II antigen gamma chain	0.673	8.84 ± 3.44
MHC class II DPw3-α-1 chain SB	0.538	7.02 ± 2.18
Complement component 4B (C4B)	0.884	6.90 ± 3.52
MHC class II, DR beta 5 (HLA-DRB5)	0.267	4.82 ± 0.76
DEF subunit LMP7 proteasome subunit, beta type, 8	0.541	4.31 ± 1.35
α-2-macroglobulin (A2M)	0.466	4.31 ± 1.16
I factor (complement) (IF)	0.384	4.24 ± 0.94
MHC class II antigen (HLA-DRB1)	0.259	4.09 ± 0.61
MHC class II, DP beta 1 (HLA-DPB1)	0.422	3.98 ± 0.97
Pre-B-cell colony-enhancing factor (PBEF)	0.238	3.24 ± 0.44
1 fibrinogen, gamma polypeptide	0.317	3.15 ± 0.58
Complement component 1,r	0.464	3.02 ± 0.81
MHC class II, DM alpha	0.353	2.69 ± 0.55
Complement-c1q tumor necrosis factor-related protein	0.481	2.68 ± 0.75
HLA class I heavy chain (HLA-Cw* 1701)	0.412	2.57 ± 0.61
MHC HLA-B39	0.271	2.21 ± 0.35
HLA-B, allele A*2711,	0.288	2.15 ± 0.35
MHC class I HLA B71	0.267	2.10 ± 0.32
MHC class I, C	0.244	2.09 ± 0.30
β2 microglobulin (HLA-G2.1)	0.325	2.09 ± 0.39
Similar to human leukocyte antigen C α chain	0.175	1.89 ± 0.19
MHC class I, E (HLA-E)	0.070	1.87 ± 0.08
Cw1 antigen	0.204	1.81 ± 0.21
Transcription factors		
Zinc finger protein 6 (CMPX1)	0.385	3.79 ± 0.84
CCAATenhancer binding protein (CEBP), delta	0.139	3.59 ± 0.29
Interferon, gamma-inducible protein 16 (IFI16b)	0.331	3.23 ± 0.62
Cbpp300-interacting transactivator 1 (CITED1)	0.322	3.13 ± 0.58
Mad-related protein (MADR1)	0.482	2.59 ± 0.72
Activating transcription factor 3 (ATF3)	0.217	2.44 ± 0.31
Insulinoma-associated 1 (INSM1)	0.278	2.30 ± 0.37
v-fos, FBJ osteosarcoma viral oncogene homolog	0.109	2.20 ± 0.14
Novel MAFF (v-maf oncogene family)	0.124	2.01 ± 0.14
STAT3	0.190	1.89 ± 0.36
B-cell CLL lymphoma 6 (zinc finger protein 51, BCL6)	0.201	1.69 ± 0.21
STAT1	0.081	1.48 ± 0.07

Table 2 (Continued)

Genes decreased upon LIF withdrawal	CV	Fold change
Neural proteins		
Ephrin-A1 (EFNA1)	0.637	5.93 ± 2.18
Chimerin	0.569	4.80 ± 1.58
Glial fibrillary acidic protein (GFAP)	0.411	3.58 ± 0.85
Neuropeptide Y (NPY)	0.501	3.13 ± 0.91
Neurofilament, light polypeptide (68 kDa)	0.138	3.05 ± 0.24
Monoamine oxidase B (MAOB)	0.396	2.78 ± 0.64
Dihydropyrimidinase-like 3 (DPYSL3)	0.172	2.27 ± 0.23
Epithelial membrane protein 3 (EMP3)	0.502	2.21 ± 0.23
Growth associated protein 43 (GAP43)	0.147	1.97 ± 0.17
Secretogranin II (chromogranin C, SCG2)	0.138	1.88 ± 0.15
Peripheral myelin protein 2 (PMP2)	0.298	1.71 ± 0.29
Signal transduction		
Insulin-like growth factor binding protein 7 (IGFBP7)	0.389	3.16 ± 0.71
Tyrosine phosphatase type IVA, member 3 (PTP4A3)	0.171	2.99 ± 0.29
Serum glucocorticoid regulated kinase (SGK)	0.260	2.89 ± 0.43
Pleckstrin homology-like domain, family A, member 1	0.179	2.07 ± 0.21
G protein-coupled receptor, family C, group 5, member B	0.069	1.74 ± 0.07
Tumor necrosis factor receptor superfamily, member 1 A	0.105	1.71 ± 0.10
Secreted frizzled-related protein 1 (SFRP1)	0.138	1.67 ± 0.13
RGL protein (RGL)	0.206	1.65 ± 0.20
ER protein processing		
Sorting nexin 10 (SNX10)	0.462	6.63 ± 1.77
1,2-alpha-mannosidase IC (HMIC)	0.249	2.21 ± 0.32
Protein disulfide isomerase-related (PDIR)	0.150	2.26 ± 0.20
Tyrosylprotein sulfotransferase 1 (TPST1)	0.041	1.70 ± 0.04
Cell adhesion/cytoskeleton-linked		
Protocadherin 8 (PCDH8)	0.434	5.34 ± 1.34
S100 A10 (calpactin I, light polypeptide, p11)	0.567	3.42 ± 1.12
CD44 antigen	0.205	3.37 ± 0.40
Podocalyxin-like (PODXL)	0.722	3.09 ± 1.29
Ankyrin 3, node of Ranvier (ankyrin G)	0.390	2.77 ± 0.62
pallid (mouse) homolog, pallidin	0.234	2.56 ± 0.35
Myosin VB	0.235	2.04 ± 0.28
Lectin, galactoside-binding, soluble, 3 (galectin 3)	0.147	1.97 ± 0.17
Adaptor-related protein complex 1, sigma 2 subunit (AP1S2)	0.082	1.96 ± 0.09
Kangai 1 (CD82 antigen, R2 leukocyte antigen)	0.206	1.89 ± 0.23
Glypican 1	0.190	1.89 ± 0.21
Lipocortin 2 pseudogene (LIP)	0.289	1.87 ± 0.31
Annexin A2 (ANXA2)	0.172	1.80 ± 0.17
RBX membrane. protein band 4.1-like 3	0.176	1.76 ± 0.18
Integrin associated protein (CD47)	0.178	1.68 ± 0.17
S100 calcium-binding protein A11 (calgizzarin, S100A11)	0.235	1.66 ± 0.23
Cell adhesion molecule with homology to L1CAM	0.141	1.63 ± 0.13
Miscellaneous		
Ribosomal protein L4	0.332	17.03 ± 3.27
Solute carrier family 4, anion exchanger, member 3	0.941	4.11 ± 2.24
Phorbol-12-myristate-13-acetate-induced protein 1	0.247	2.95 ± 0.42
Cathepsin C	0.104	2.59 ± 0.16
Gene similar to cerebellin precursor	0.413	2.54 ± 0.61
Cathepsin D (lysosomal aspartyl protease)	0.273	2.53 ± 0.40
Interferon induced transmembrane protein 2 (1–8D) IFITM2	0.125	2.47 ± 0.18

Table 2 (Continued)

Genes decreased upon LIF withdrawal	CV	Fold change
ATPase, Class VI, type 11 A	0.150	2.26 ± 0.20
Protein S (alpha) (PROS1)	0.109	2.20 ± 0.14
Pyruvate dehydrogenase phosphatase (PDP)	0.460	2.13 ± 0.57
Phospholipid scramblase 1	0.147	2.11 ± 0.18
Photolyase, cryptochrome 1 (photolyase-like)	0.070	2.00 ± 0.08
Lectin, galactoside-binding, soluble, 3 binding protein	0.147	1.97 ± 0.17
Ring-IBR-ring domain containing protein Dorfin	0.193	1.76 ± 0.20
Butyrobetaine (γ), 2-oxoglutarate dioxygenase (BBOX1)	0.286	1.75 ± 0.29
Complement cytolysis inhibitor (CLI) clusterin	0.169	1.72 ± 0.17
Coagulation factor III (thromboplastin, tissue factor, F3)	0.072	1.52 ± 0.06
Genes increased upon LIF withdrawal		
Neurexin I-alpha protein	0.579	11.19 ± 3.75
Protein tyrosine phosphatase, receptor type C	0.407	3.69 ± 0.87
RGC32 protein (RGC32)	0.304	3.29 ± 0.58
Spondin 1 (f-spondin)	0.409	2.96 ± 0.70
Highly similar to CLP	0.312	2.11 ± 0.38
Cyclin D1 (PRAD1)	0.206	1.99 ± 0.24
Activated leucocyte cell adhesion molecule	0.082	1.96 ± 0.09
Cysteine dioxygenase, type 1 (CDO1)	0.126	1.75 ± 0.13
Epidermal growth factor receptor (v-erb-b)	0.179	1.68 ± 0.17
Cystatin C (CST3)	0.206	1.65 ± 0.20
ATPase Na, K transporting, α2 poypeptide (ATP1A2)	0.007	1.52 ± 0.01

Gene expression profiles from ltNSC^{ctx} grown long term in EGF/LIF were compared with those withdrawn from LIF for 9 days using Affymetrix U133 microarrays, and significant increases or decreases were determined. Significance was determined by rank, fold-change, and coefficient of variation (CV). Genes were clustered through literature review into functional groups.

There were also significant reductions in a range of immune response genes, including both major histocompatibility complex I and II (MHC-I, MHC-II). GFAP is known to be modulated by this cytokine in the developing brain (Koblar *et al.* 1998), and its expression was significantly reduced in ltNSC^{ctx} when LIF was withdrawn. A large number of unidentified ESTs were decreased when LIF was withdrawn. The list of ESTs exhibiting LIF-dependent changes in expression levels is accessible in the supplementary data Table S3 (available online at http://www.waisman.wisc.edu/genechipdata/).

MHC-I, CD44 and GFAP protein levels are dynamically modulated by LIF

To validate that the changes in gene expression were reflected at the protein level and to determine if a particular gene was expressed in only a subset of cells within the neurospheres, we performed immunocytochemical and western blot analysis on selected gene products. ltNSC^{ctx} grown long term in LIF, withdrawn from LIF for 9 days and then re-exposed to LIF for an additional 3 days, were dissociated, plated for 1 h, fixed and immuno-stained for MHC-I and CD44. Every cell from the EGF/LIF cultures expressed high levels of both MHC-I (Figs 5a and b) and CD44 following acute plating (Figs 5d and e). As predicted from the oligonucleotide array analysis, the number of cells expressing each protein decreased significantly following LIF withdrawal (MHC-I to 36% and CD44 to 27%) and increased again following LIF re-application [MHC-1 to 49% (Figs 5a and c) and CD44 to 46% (Figs 5d and f)]. Immunoblots for GFAP revealed striking reductions following LIF withdrawal within intact spheres, which increased following 3 days of re-application (Fig. 6a). Following 1 hour of plating dissociated ltNSC^{ctx} grown in EGF/LIF, greater than 90% of the cells expressed high levels of GFAP (Figs 6b and c). Following LIF withdrawal, GFAP down-regulated in 30% of cells (Fig. 6d) but maintained full nestin expression (Fig. 2a). There was a significant increase in GFAP expression in cells following 3 days of LIF re-application (Fig. 6e), demonstrating a dynamic effect of LIF within individual cells at the protein level.

Discussion

The routine growth of large numbers of human neural stem cells is a pre requisite for the use of such cells for either cell therapy or drug screening. ltNSC^{ctx} cultures are comprised of over 92% nestin-positive cells and as such represent a

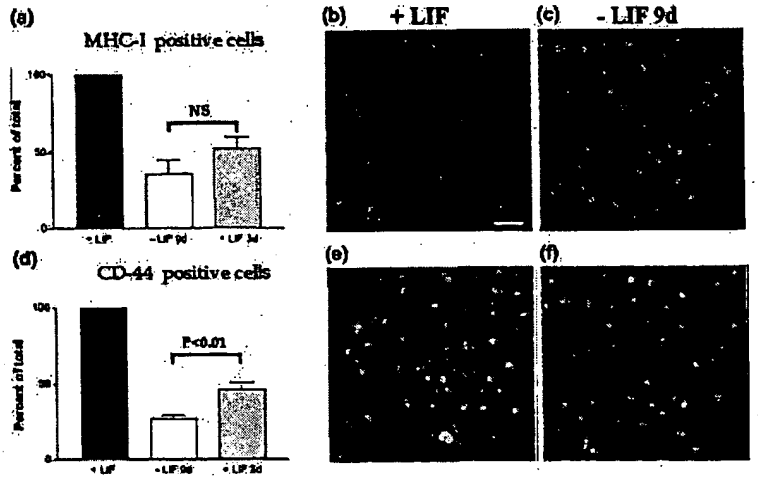

Fig. 5 LIF affects MHC-1 and CD44 expression in ltNSC^{ctx}. ltNSC^{ctx} cultures (K052, K054, K066) were grown for a total of 50 weeks in media supplemented with EGF for the entirety of culture and LIF supplementation starting at 20 weeks, withdrawn from LIF for 9 days followed by re-exposure to LIF for 3 days. Whole spheres were dissociated, plated for 1 h before fixation, and immuno-stained for MHC-1 (red), CD44 (green) and Hoechst-labeled nuclei (blue). The percentages of MHC-positive (a) and CD44-positive cells (d) were determined. There were significant decreases in the percentage of labeled cells upon LIF withdrawal for both MHC-1 and CD44. Re-application of LIF significantly increased the number of CD44-positive cells. Photomicrographs of MHC-1 positive cells (b) and CD44-positive cells (e) grown long-term in EGF/LIF compared with that of cells withdrawn from LIF for 9 days (c, f) show a decrease in intensity of immuno-staining as well as a decrease in the number of labeled cells. Bar: 50 μm.

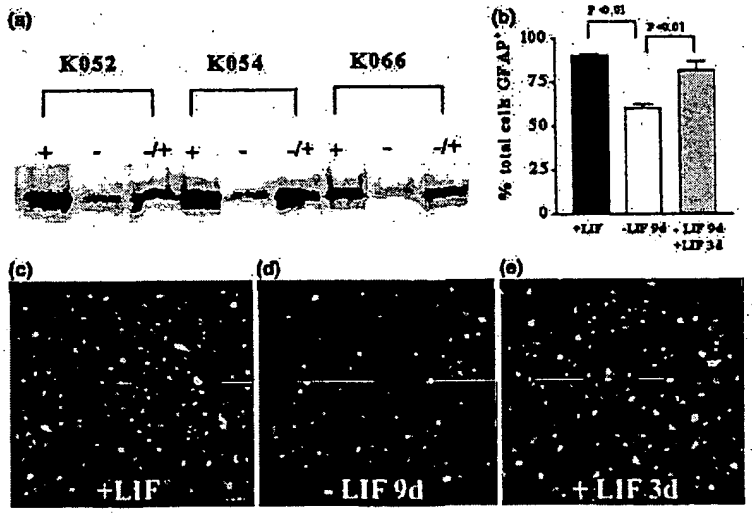

Fig. 6 GFAP expression is dynamically regulated by LIF. ltNSC^{ctx} cultures (K052, K054, K066) were grown for a total of 50 weeks in media supplemented with EGF for the entirety of culture and LIF supplementation starting at 20 weeks, withdrawn from LIF for 9 days followed by re-exposure to LIF for 3 days, and then prepared for western analysis or immunochemistry. Protein from cell-free lysates from each treatment was transferred to immobilon membrane and immuno-blotted against anti-GFAP (a). Whole spheres were dissociated, plated for 1 h before fixation, immuno-stained for GFAP (red), Hoechst-labeled nuclei (blue), and the percentage of GFAP-positive cell was determined (b). Photomicrographs of cells grown long term in EGF/LIF (c), withdrawn from LIF for 9 days (d) or withdrawn from LIF for 9 days and re-exposed to LIF for 3 days (e) confirm the changes in GFAP labeling. Bar: 100 μm.

relatively pure undifferentiated population although, within this group, there are likely to be a number of cellular subtypes. When LIF was withdrawn, the growth rate decreased but was restored by LIF re-application. This data shows that the growth rate of these cells is dynamically regulated by LIF. The finding that the number of Tuj1-positive cells and nestin-positive cells remained unchanged after LIF withdrawal suggests that LIF is regulating some aspect of the nestin-positive cell population that allowed faster growth and continual self-renewal, rather than preventing differentiation into post-mitotic neurons. ltNSC^{ctx} cultures could be maintained for extended periods of time without losing the ability to generate high numbers of neurons, indicating that neurogenesis was unimpaired even after prolonged exposure to EGF and LIF. Therefore, we consider these long-term EGF/LIF neurosphere cultures an ideal candidate for determining basal gene expression in NSC.

The characterization of gene expression profiles described in this paper gives investigators a database to explore the basic biology of these cells and a guide for experimental design. For instance, patterns of receptor expression informs directly as to which growth factors may be important for their subsequent differentiation. We emphasize that if neurospheres are generated from different brain regions other than cortex (as used in the current study) patterns of gene expression are likely to be different due to regional specification events early in development (Hitoshi *et al.* 2002; Ostenfeld *et al.* 2002a) (Svendsen *et al.*, unpublished observations). Furthermore, as cells divide in culture, they appear to undergo temporal changes in differentiation potential that may reflect their natural *in vivo* development (Qian *et al.* 2000; Temple 2001). Therefore, during early phases of expansion, there may be many temporal changes occurring in gene expression that reflect normal development. In the current study, we performed oligoneucleotide microarray analysis on the long-term self-renewing cells in a relatively stable phase of growth that may have resulted in far more stable gene expression profiles (between 30 and 110 PD). Given the complexity and scale of human cortical development, this time period may represent the normal expansion phase required to generate this brain region. Alternatively, we may have isolated a cell that is equivalent to the adult neural stem cell that resides in the sub-ventricular zone (Doetsch *et al.* 1999). It is of interest that, even in the long-term presence of LIF, the growth rate of these ltNSC^{ctx} diminishes after 110 PD. This observation would perhaps favor the first hypothesis, suggesting that this culture represents a somatic type of stem cell with extended, but not unlimited, capacity for self-renewal. Another possibility is that ltNSC^{ctx} undergo senescence due to telomere erosion (Harley *et al.* 1990). We have previously reported that telomerase levels are very low in human neurospheres (Ostenfeld *et al.* 2000) and confirm this finding in the current study where telomerase was called absent (Table 1). Interestingly, exposure to LIF did not enhance telomerase expression, suggesting that the LIF-dependent increase in growth rates was not due to greater telomerase activity, but its absence may explain why our cultures finally senesce. However, telomerase is only expressed at very low levels in hNSC even at early time points, and our data may simply reflect a lack of sensitivity to detect very low levels of RNA. We are currently looking in more detail at the relationship between telomerase and longevity in these cultures using more sensitive techniques.

Recent descriptions of gene expression patterns within stem-cell populations are starting to define their molecular profiles (Geschwind *et al.* 2001; Ivanova *et al.* 2002; Ramalho-Santos *et al.* 2002). From these studies, it is clear that while a few genes overlap between stem cells isolated from different tissues, there are many more which are specific to each stem-cell population. Ivanova *et al.* generated neurospheres from the embryonic lateral ventricle striatum, while Ramolho-Santos derived their neurospheres from the adult mouse sub-ventricular zone. As discussed previously, there are significant differences in gene expression between both regions and species making comparisons with these papers very difficult. However, we did see expression of a few genes in our cultures that identified as stem-cell specific in common by those groups. These genes include Fyn, Slit2, and insulin-like growth factor binding protein 2. When we looked for genes specifically associated with neural development, but not modified by LIF, there were very interesting patterns. It was of interest that none of the *Wnt* genes were expressed within the neurospheres. *Wnt* has recently been show to antagonize neural development in mouse ES cells (Aubert *et al.* 2002). In some cases, these findings may be very relevant to future studies looking at differentiation or proliferation of these cells. For example, the FGF receptors FGFR1, and FGFR3 were expressed but not FGFR2, 4 or 5. The expression of FGF-2 and its receptor FGFR1 may imply paracrine or autocine effects of this mitogen on the growth of these EGF-responsive cells. Similarly, platelet-derived growth factor receptor (PDGFR) alpha, but not beta, was expressed. PAX6, which is known to play a role in cortical neurogenesis, was also expressed (Estivill-Torrus *et al.* 2002) but not PAX3, 4 or 8. Interestingly, a number of genes that we expected to see expressed at high levels were not expressed. For example, musashi1 has been associated with both rodent and human neural stem cell populations (Kaneko *et al.* 2000; Keyoung *et al.* 2001). As Keyoung *et al.* examined musashi1 in short-term human cultures only, it is possible that this RNA-binding protein is down-regulated after the longer growth periods used in this study.

LIF is vital for long-term self-renewal within ltNSC^{ctx} cultures. Therefore, we assessed which genes might be effected by LIF and whether they may be expected to have a role in that long-term growth. We found that the expression

of many genes was altered by LIF, but whether a specific gene is directly regulated by LIF or is effected by a downstream event is difficult to determine from this type of analysis. However, it is clear that LIF can, either through direct or indirect routes, affect specific patterns of gene expression in these cultures.

Some interesting genes were increased following LIF withdrawal. The increase in EGF receptor expression suggests a negative feedback system in which lack of LIF signaling induces increased production of this receptor. Similar mechanism may explain the increases in cystatin C, a factor required for long-term growth of FGF-2 responsive rodent neural stem cells (Taupin *et al.* 2002). Significant increases in cyclin D following LIF withdrawal was perhaps unexpected, as it has been suggested to represent one of the possible markers for stem cells in different tissues (Ivanova *et al.* 2002; Ramalho-Santos *et al.* 2002). However, it has previously been shown that cyclin D expression also increases following LIF withdrawal from mouse ES cells (Savatier *et al.* 1996). It is therefore possible that cyclin D is a marker for division in more lineage-restricted EGF-responsive, LIF-unresponsive neural precursor cells in our culture system.

The LIF-dependent changes in cell adhesion molecules might be very relevant to the growth of cells as neurospheres. In particular, the ephrin (EFN) family showed an interesting pattern of expression. EFN A2, A3, A4, A5 and B3 were not expressed in the neurospheres under any condition, EFN B2 was expressed but not changed by LIF, and EFN A1 showed a 5.93-fold decrease following LIF withdrawal. As EFN A1 is known to play a role in the three-dimensional growth of carcinoma cells, perhaps by alleviating contact inhibition (Potla *et al.* 2002), it is a possible contributor to the increased three-dimensional growth of the neurospheres. A number of important cell adhesion molecules were also modulated by LIF. Protocadherin 8 is a member of the cadherin family expressed in embryonic neural tissues and associated with synaptic structure (Frank and Kemler 2002) and was decreased 5-fold following LIF withdrawal. Glypican 1 and CD44 were also reduced, and both represent important cell-adhesion molecules directly connected with cell/cell interactions at the cell (Lander *et al.* 1996).

LIF was found to positively regulate MHC, CD44 and GFAP gene and protein expression levels. Cytokines, including interferon and IL-1, can induce MHC-II expression in astrocytes, (for review see Dong & Benveniste 2001), and neural stem cells derived from the rodent (McLaren *et al.* 2001). LIF has also been shown to induce HLA-G in choriocarcinoma cells (Bamberger *et al.* 2000), suggesting a role in immune response modulation. We do not yet know the function of increased MHC expression in ltNSC^{ctx} following LIF administration, but this may be important when considering rejection issues following transplantation of these cells into the damaged brain. The cell surface proteoglycan CD44 binds hyaluronan and has been implicated in a wide range of cellular functions (Lesley *et al.* 1993). CD44 has been detected in dorsal, but not ventral, progenitor cells in the rat spinal cord as early as E13.5 (Liu *et al.* 2002). CD44 was expressed in A2B5+ cells isolated from E14.5 rat neural tissue, but not in embryonic neural cell adhesion molecule (E-NCAM)+ cells, suggesting that CD44 may be a marker for astrocyte progenitors. (Luo *et al.* 2002). In addition, keratinocytes from CD44 knock-out mice fail to proliferate in response to HB-EGF (Kaya *et al.* 1997), implying a role in cell proliferation for this molecule. Studies using functional blocking experiments are currently underway to establish the effects of CD44 and MHC on the long-term growth of ltNSC^{ctx}.

The finding that GFAP was regulated by LIF in ltNSC^{ctx} is in agreement with a number of studies showing that this intermediate filament can be regulated via gp130 and the JAK-STAT signaling pathway (Bonni *et al.* 1997; Nakashima *et al.* 1999). Neural stem cells generated from LIF knock-out mice also show significant reductions in GFAP expression following differentiation, suggesting that astrocyte production may be under the control of LIF. LIF or CNTF may direct NSC towards an astrocytic fate (Bonni *et al.* 1997; Koblar *et al.* 1998). However, these reports described neural cells which were undergoing differentiation in the absence of mitogens, and, once generated under these conditions, these astrocytes generally maintained GFAP expression but down-regulated nestin. These treatments may cause the cells to be committed solely to becoming astrocytes (Johe *et al.* 1996; Bonni *et al.* 1997; Caldwell *et al.* 2001). We show here that more than 90% of nestin-positive ltNSC express GFAP in the presence of LIF, and that there is rapid down-regulation of GFAP following LIF withdrawal. Therefore, LIF is also able to regulate GFAP expression within the growing stem-cell population without affecting nestin expression. Interestingly, GFAP/nestin-positive cells resembling astrocytes have been identified as neural stem cells in the adult mammalian brain (Garcia-Verdugo *et al.* 1998; Doetsch *et al.* 1999) and are direct descendants of embryonic radial glial cells in the embryo (Malatesta *et al.* 2000; Miyata *et al.* 2001; Noctor *et al.* 2001, 2002). The expression of high levels of GFAP in nestin-positive ltNSC capable of extensive self-renewal and neurogenesis, provides further evidence that a similar cell may exist within the human CNS. The molecular profile described in this report may lead to a better understanding of factors that control the fate of this GFAP-positive stem cell.

In this study, we show that a LIF/EGF-responsive human neural stem cell can be maintained for long periods in culture and describe basal gene expression in these cultures. Furthermore, we identify a number of genes were dynamically regulated by LIF. Clearly the limitations of detection of the microarray system preclude the complete description of gene expression in neural stem cells. However, given this

caveat, we still feel that this database will be of value to others who are interested in human neural stem cells, and further investigation of the genes responding to LIF may lead to a better understanding of the mechanisms underlying their long-term self renewal. In addition, the capability of NSC to survive and integrate into the damaged brain (for review, see Svendsen & Caldwell 2000) underscores the great therapeutic potential for these cells. The full characterization of these cells is a vital prerequisite to clinical transplantation, and this report is a first step towards this goal.

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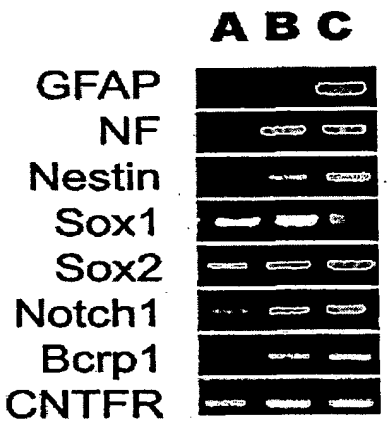
Exhibit K



A Is a Passage 0 isolate of NPC's from the earliest stage of differentiation from hES cells.
B: Is a Passage 2 population of NPC's derived from hES cells.
C: Is a Adult cortex progenitor cells (which are very likely the same as those isolated by Carpenter from the forebrain).